

Olaf Wraae

Kinetic Studies of the Lithium Ion in Rat Brain *in vitro* and *in vivo*



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Jørgen Ringsted
h.a.dec.





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The present thesis includes results from the following previous publications:

Wraae, O., Hillman, H. & Round, E.: The uptake of low concentrations of lithium ions into rat cerebral cortex slices and its dependence on cations. J. Neurochem. 1976, 26: 835 - 843

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P R E F A C E

The experimental work for the present thesis was carried out from 1973 to 1976 at the Department of Human Biology, University of Surrey, England, during my tenure of a Research Scholarship from the University of Aarhus. Some supplementary experiments (for parts of Chapter Seven) were done at the University of Odense during the Spring of 1977.

Though formally presented as the work of one person, a thesis is also the result of other people's thoughts, suggestions and practical help. I have tried to acknowledge this by mentioning the main contributors below.

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BASIS AND OUTLINE OF STUDY

Since the introduction of Li^+ into psychiatry (Cade, 1949) the use of lithium salts against manic depressive disease has been universally accepted, largely due to careful clinical trials in this country (Schou et al., 1954; Baastrup et al., 1970). The range of clinical uses of Li^+ has been assessed recently (Schou, 1978b); it has been extended considerably since Cade's discovery, as has the interest in the biology and pharmacology of Li^+ , which is reflected in the enormous increase in frequency of publications (see e.g. Schou, 1976b, Fig.1). The intensified research has demonstrated effects of Li^+ on a large number of biochemical systems.

The physiochemical properties of Li^+ are close to those of the four biologically important cations, K^+ , Na^+ , Ca^{2+} and Mg^{2+} , and Li^+ interacts with a number of cellular systems whose activity is influenced by these cations. Li^+ might thus affect cellular carriers or other sites occupied by one of these ions or it might interfere with the ion channels of cell membranes (Bunney & Murphy, 1976, p. 132 - 164). Such phenomena should be observable in the kinetics of Li^+ by means of saturation and inhibition studies (Christensen, 1975, p. 111 - 113). Also, the brain is likely to be the target organ of the antipsychotic effect of Li^+ . Hence the attempts of the present thesis to investigate the kinetics of Li^+ in cerebral tissue. As will be discussed in Chapter Four and Six, the Nernst equation (e.g. Christensen, 1975, p. 85) would predict a much higher intra/extracellular ratio for a cation like Li^+ than the relatively even distribution between tissue and extracellular fluids observed in most tissue.

Therefore, factors changing the ratio might suggest mechanisms causing the ratio. The ratio would also point to asymmetry of transport mechanisms and the need to study influx and efflux separately. On the other hand, it was felt intuitively that it would be more profitable to find out what makes Li^+ associate with the cells, rather than what keeps it out, and also simpler from a technical point of view. Accordingly, the emphasis in the experiments of the present thesis has been on the uptake.

In the uptake of Li^+ from the blood stream to the brain of the living animal there is more than one anatomical barrier of diffusion between the blood and the cerebral cells. Therefore, the kinetics of Li^+ is studied in two different models of the brain, one in the absence of the blood-brain barrier, and one in which it is present.

In the cerebral slice, the neuronal and glial membranes are in direct contact with an adjustable incubation medium. The tissue slice was introduced by Warburg (1923) as a preparation for measurement of respiration and metabolism of glucose in tumours, but its usefulness in transport studies has since been tested in numerous experiments. The properties of the cerebral slice have been investigated first and foremost by McIlwain and co-workers. It has been shown to maintain to some extent ionic gradients, membrane potentials, susceptibility to electrical and chemical stimulation, metabolism of high energy phosphates and other characteristics of the living brain (McIlwain & Bachelard, 1971, p. 61 - 97). The incubation medium used in most experiments with cerebral slices bears the names of Krebs and Ringer. Observing the influence on the heart beat of various salt solutions, Ringer arrived at a composition roughly similar to that used in the present experiments. His main contribution, namely that of including calcium in his medium, was based on an accidental finding whose implications he recognized (Ringer, 1883; Dale, 1948, p. 453). Warburg used Ringer's solution, which contained bicar-

bonate, and added CO_2 to the aerating gas, thus improving the incubation medium with a physiological buffer (Warburg, 1926, p. 109 - 112). The later modifications of the media implied the addition of various oxidizable substrates. They were based on analytical data from blood plasma and the ability to support respiration of the tissue (Krebs, 1950). In the standard medium of the present thesis (Table 2.2), glucose is used as the only oxidizable substrate as this has been found to be adequate for most purposes (McIlwain & Rodnight, 1962, p. 137 - 139; McIlwain & Bachelard, 1971, p. 70 - 72).

Cerebral slices have often been used to examine various effects of Li^+ on brain biochemistry. In a few experiments, an interaction of Li^+ with K^+ and Na^+ has been observed in cerebral slices (Pappius et al., 1958, Israel et al., 1966, Kjeldsen et al., 1973). Only those of Kjeldsen et al. were designed specifically to examine this question, and only they used concentrations of Li^+ in the media comparable to serum values of patients under Li^+ treatment. Hitherto, in cerebral slices an interaction of Li^+ with Ca^{2+} or Mg^{2+} or its influx or efflux does not seem to have been studied. With one exception (Chapter Five), which is discussed later, the concentrations of Li^+ in the media of the present experiments using cerebral slices are low (0.5-2 mM), thus justifying comparisons with observations in vivo.

Partly due to its toxicity and the simplicity of its measurement, Li^+ is still the only psychopharmacological compound that is monitored regularly in the serum of patients. For the same reasons, Li^+ is probably one of the drugs whose pharmacokinetics in whole animals has been most thoroughly investigated. The slow penetration of Li^+ into the brain after acute administration to rats was demonstrated early (Davenport, 1950; Schou, 1958). Recent studies have confirmed these findings, and have also shown that the elimination from the brain is equally slow compared to that from the serum (Frazer et al., 1973; Ebadi et al., 1974; Lehmann, 1975; Mukherje et al., 1976).

Presumably, the unfavourable correlation between the concentration of Li^+ in the brains and sera of rats in the kinetic experiments above can be extrapolated to patients. Therefore, it would be natural to look for parameters that would reflect the cerebral concentration of Li^+ better than does the rapidly changing serum Li^+ and that were available in patients also. From their data, Frazer et al. suggested Li^+ in red cells, thereby triggering an intensive research, which will be discussed in the final chapter. In the present experiments (Chapter Seven), in which the kinetics of low concentrations of Li^+ in the serum and the brain of rats are investigated, Li^+ in the cerebrospinal fluid is chosen as the third parameter. It has been shown that this parameter correlates well with the serum values in patients in steady state with respect to Li^+ . Also, cerebrospinal fluid is the extracellular fluid that is generally assumed to reflect most reliably the penetration of drugs into the brain. Finally, it must be the nearest anatomical correlate to the incubation fluid of the cerebral slice. Simultaneous measurements of the kinetics of Li^+ in the brain, cerebrospinal fluid and serum do not seem to have been performed earlier.

The results of the experiments of this thesis are presented in the following six chapters.

Chapter Two deals with the fundamental methodological problems in the kinetic experiments of chapters three to six. The assessment is based on a review of the relevant literature and a number of control experiments (seventeen tables). The advantages of surrounding the cells of the brain with a fluid whose composition can be changed at will are obvious. However, the advantages gained in simplification might be lost because of artifacts produced by the isolation. Therefore, the relative importance is assessed of the large number of variables in the isolation and incubation of cerebral tissue, such as the delay between the killing of the animal and the incubation, the cutting of the slices, the thickness of the slices, the evapora-

tion, pH changes and oxygenation of the medium. Formalin fixed slices are employed as inert controls of metabolic effects.

In kinetic experiments it is an advantage that Li^+ is not metabolized, and a disadvantage that there are no radioactive isotopes of Li^+ with a half-life longer than a few seconds. Though it has been possible by means of neutron activation to use autoradiographic techniques in Li^+ measurements (Zajcik *et al.*, 1972; Thellier *et al.*, 1976; Carpenter *et al.*, 1977) the equipment required clearly makes it an exclusive method. Compared with radioactive methods, photometric measurements are not very sensitive. Interference from other elements and from organic compounds, and artifacts due to the preparation before the measurements, may assume great significance when low concentrations of Li^+ are measured in the presence of tissue with a changing composition. Section (ii) of the chapter deals with the measurement of Li^+ , K^+ and Na^+ in cerebral tissue.

In cerebral slices, solutes transferred from the medium to the cells have to pass through not only the cell membrane but also an extracellular space. Furthermore, an amount of medium-like fluid adheres to cerebral slices after they have been incubated. Therefore, in order to measure the concentration of solutes in the cells, the amount of solids, extracellular fluid and adhering medium must be known. Section (iii) contains a review of the literature by means of which the reliability of various methods of measuring these parameters is assessed. The reasons for referring concentrations of solutes to incubated weight are given, and the consequences of expressing the results in this way are discussed.

The previous sections of Chapter Two deal with sources of systematic error in experiments with cerebral slices. However, due to the many uncontrollable variables and the small number of samples in each experiment, random error is a problem in

assessing the results of this thesis. Therefore, statistical methods are an essential part of the thesis. In section (iv) the assumptions underlying the use of some common statistical tests are examined, especially in connection with the combination of results and multiple comparisons. The subjective nature of many decisions in statistics is stressed. Chapter Two ends with a short section on general problems in the interpretation of results.

In Chapter Three the evidence of saturation in the uptake of low concentrations of Li^+ is examined using the conventional assumptions about initial uptake, namely that the concentration of the solute in the extracellular fluid is that of the medium and that there is no significant back-flow during brief periods of uptake. In some cases the slow diffusion of a solute into the extracellular space may invalidate these assumptions (Lund-Andersen & Kjeldsen, 1976). Therefore, the observed rates of uptake are compared with that of free diffusion. The net uptake is measured for later comparison with the duration of the uptake in vivo; its dependence on metabolism is examined, and interpretations are discussed; finally, it is subjected to a compartmental analysis in an attempt to find evidence of differences between the two main types of cells with respect to the uptake of Li^+ .

The concentration ratio of Li^+ at steady state between the slices and the medium is investigated in Chapter Four. The effects of various low concentrations of Li^+ and of metabolism on the ratio are examined. The influence of various low concentrations of K^+ , Na^+ , Ca^{2+} and Mg^{2+} on the Li^+ in the slices is systematically tested. On the basis of earlier findings by other authors (Kjeldsen et al., 1973) the influence of low concentrations of Li^+ in the medium on the K^+ and Na^+ of the slices are also examined.

The concentrations of Li^+ used in the experiments of Chapter

Three and Four correspond to the serum levels of patients treated with Li^+ . This narrow range of concentrations limits the possibility of interpreting kinetic findings. The observations of Chapter Four cannot be explained by a single mechanism of uptake or extrusion of Li , but a priori, inhibition of the uptake of Li^+ by K^+ , Ca^{2+} or both ions is thought likely. Therefore, the initial uptake of Li^+ is reinvestigated using the same concentrations of K^+ and Ca^{2+} as in the experiments of Chapter Four and concentrations of Li^+ up to 30 mM. Evidence of saturation and inhibition is examined by means of the Lineweaver-Burk regression; an attempt has been made to remove the bias of the method by statistical weighting. The possibility is considered that there may be a combination of saturable and passive uptake of Li^+ , as has been observed with other solutes. Published methods of correction for passive uptake are modified for the present purposes. Experiments controlling for adverse effects of changes in osmolarity, low Na^+ and high Li^+ are performed.

Chapter Six is the last chapter dealing with the kinetics of Li^+ in cerebral slices. After having obtained a steady state with the concentration of Li^+ used most frequently, 1.5 mM, the efflux is measured during the next 150 min. An efflux curve is constructed from eight experiments, and a compartmental analysis is performed. The bias caused by efflux through the extracellular space is assessed by means of Huxley's correction. The extracellular space has been estimated using the efflux data. The rate constants of efflux and influx are compared. The K^+ of the slices is measured before and after efflux of Li^+ .

The results from kinetic experiments on intact rats are presented in Chapter Seven. As mentioned earlier, measurements of Li^+ in the cerebrospinal fluid have been done because this is an extracellular fluid in direct contact with the

cells of the brain and available in patients also. Therefore, the findings can be compared with results of experiments with cerebral slices as well as with data from patients. The uptake and elimination after intraperitoneal injections is followed for 48 h after which Li^+ is no longer measurable. The β -phase might not occur within this period in the elimination of Li^+ from the brain because of a high affinity of Li^+ for cerebral tissue, but it is usually seen earlier in elimination from steady state. Therefore, Li^+ is also measured in the brain, cerebrospinal fluid and serum for 48 h under these conditions. As each group of three points on the kinetic curves is represented by one individual, the experimental scatter is considerable. It is reduced by relating concentrations in the brain and cerebrospinal fluid to serum concentrations.

General references: There are references that it may be found difficult to cite on any particular subject, but which have been useful in introducing a topic, as reference books or in providing primary sources. It is felt appropriate to mention them in this introductory chapter under the various subject headings. They are given in full detail in the reference section.

Biochemical calculations: Dawes (1972)

Biological transport: Stein (1967); Davies (1973)

Cerebral slices: Marchbanks (1970)

Enzyme kinetics: Andersen & Jørgensen (1969 & 1970)

Lithium: Schou's review (1957) and later bibliographies. Gershon & Shopsin (1973); Johnson (1975); Johnson & Johnson (1978).

Pharmacokinetics: Notari (1975); Kudsk (1976)

SUMMARY

1. Li^+ interacts with a large number of cellular systems

whose activity is influenced by K^+ , Na^+ , Ca^{2+} and Mg^{2+} . It is likely that Li^+ might affect cellular sites occupied by these ions. This interaction should be observable in kinetic studies.

2. The aim of the present thesis is to investigate the kinetics of Li^+ in vitro in a composite cerebral tissue without the blood-brain barrier and in the intact brain after administration of Li^+ in vivo.

3. The cerebral slice has been chosen for the studies in vitro. Its properties and those of the incubation medium are reviewed. Earlier kinetic studies of Li^+ in this preparation are few. Interaction with Ca^{2+} or Mg^{2+} and influx or efflux do not seem to have been examined before.

4. Simultaneous measurements of the kinetics of Li^+ in the brain, cerebrospinal fluid and serum do not seem to have been performed earlier. Cerebrospinal fluid has been chosen because of its availability in patients and animals and as the nearest anatomical correlate to the incubation fluid of the cerebral slice.

5. The rationale and outline of chapter two to seven are presented.

ASSESSMENT OF RESULTS OBTAINED BY MEANS OF CEREBRAL SLICES

In the historical outline of the last chapter, a general description of the tissue slice was given and similarities between the living brain and the isolated preparation were mentioned. The aim of the present chapter is to examine critically 1) the differences between a living brain and a cerebral slice 2) the accuracy of the present measurements 3) ways of expressing the concentration of a substance in a cerebral slice 4) the use of statistical tests in the present experiments and 5) interpretation of results in general. Thus, the intention in this chapter is to follow the whole procedure through the experimental and conceptual steps from the killing of the animal until the conclusions about mechanisms.

1) THE EFFECTS OF ISOLATION AND INCUBATIONCriteria of functional activity

Obviously, there is a vast difference between a human, thinking brain and a piece of rat brain in a salt solution. However, from a neurochemical point of view the species differences between mammalian cerebral tissue are small, and, obviously, the activity of the isolated tissue must be assessed in a way that is relevant to the experiments for which it is intended. Furthermore, the assessment must be easy to perform in routine experiments, and its validity must be well established by experiment. The criteria of functional activity chosen in the experiments of the present thesis were those of i) swelling ii) the concentration of K^+ and Na^+ .

i) Swelling of the slices

The uptake of fluid into the cerebral tissue is a pathological phenomenon in the intact brain, but is seen in the isolated preparation even under optimal incubation conditions. However, its degree is much dependent on the experimental circumstances. The swelling has been found to depend on:

- 1) The delay from the time when the animal is killed until incubation begins (Varon & McIlwain, 1961).
- 2) The duration of the incubation (Pappius & Elliott, 1956a; Varon & McIlwain, 1961; Hillman et al., 1974; Møller et al., 1974; Table 2.7).
- 3) The oxygenation of the slices (Pappius & Elliott, 1956a; Franck et al., 1968; Møller et al., 1974; Table 2.12).
- 4) The presence of glucose in the medium (Okamoto & Quastel, 1970; Table 2.12).
- 5) Na^+ in the medium (Elliott, 1946).
- 6) Low concentrations of K^+ in the medium (Elliott, 1946; Okamoto & Quastel, 1970; Table 4.3b).
- 7) The concentration of Ca^{2+} (Dawson & Bone, 1965; Ames et al., 1967; Table 4.4).
- 8) High concentrations of K^+ (> 20 mM) in the medium, glutamate, and the application of electrical pulses all increase the metabolism of the slices and cause increased swelling. However, these conditions were not used in the present experiments (See Hertz, 1973, p. 74 - 77, for a further discussion).

The anatomical localization of the swelling depends on the experimental conditions. Møller et al. (1974) examined this both microscopically and by means of the efflux kinetics of inulin at steady state. The swelling after incubation in a standard medium at 37°C for 1 h was located mainly extracellularly; incubation for 5 min at 37°C or for 1 h at 0°C caused less swelling, but it had the same localization. Anaerobic conditions caused considerable swelling, which was localized mainly in the cells, both the neurons and the glia, whereas 35 mM K^+ in the medium caused extreme glial swelling. The tissue has been found to be isotonic with the medium irrespective of swelling (Leaf, 1956), but the swelling can

be counteracted osmotically, in which case extracellular markers show evidence of cell shrinkage (Pappius & Elliott, 1956a).

The phenomenon of the swelling is complicated and only partly understood. However, in the assessment of experimental conditions, a high degree of swelling is generally assumed to be a sign of unfavourable conditions for the cerebral slice. Swelling may be measured by means of the weight of the solids relative to the fresh wt. (Lund-Andersen & Hertz, 1970). In the present experiments, the swelling was determined directly in all slices by weighing the slices before and after incubation. All concentrations were referred to the incubated wt., as will be discussed in section 3) of this chapter.

ii) The concentration of K^+ and Na^+

The uneven distribution of the K^+ and Na^+ between the cells and the extracellular fluid is a characteristic of the living animal cell and is dependent on metabolism for its maintenance. The concentrations of K^+ and Na^+ are well correlated with a number of other indicators of functional activity, like the resting potential, O_2 uptake, glycolysis and the concentration of high energy phosphates (McIlwain & Bachelard, 1971, p. 61 - 97; Hertz, 1973, Table VI,1), and they are easy to measure; therefore, they were used in most experiments as a check on the condition of the slices. Generally, shifts between the K^+ and the Na^+ of the slices are seen under the same experimental conditions that are associated with swelling of the slices. Some of these conditions will be discussed later in this chapter, as well as in Chapter Four and Six. The technical problems of the measurements of K^+ and Na^+ in cerebral slices are treated in section 2) of this chapter.

1A) THE EFFECTS OF ISOLATION

i) The delay in the incubation of the slices

In the incubation of tissue slices one attempts to create conditions for the tissue which are as similar as possible to those of its natural environment. Therefore, the delay from the time when the animal is killed until these conditions are established must be critical. This was found in practice in the pioneer work with tissue slices (Minami, 1923). Varon & McIlwain (1961) showed that the swelling after 1 h incubation was dependent on the time spent on handling the slices in fluids before a proper incubation was begun. Handling of the slices at 37°C caused more swelling than handling at 17 - 21°C. Bachelard et al. (1962) cut slices in situ and incubated them from 2 min to 9 min after the death of the animal. There was a highly significant linear relation between the concentration of K^+ (negative) and Na^+ (positive) and the delay in incubation. In the present experiments, the delays were recorded routinely. The average delay of some representative experiments in removal of the brain, and incubation of the first and second slice were 3 min, 7 min and 10 min, respectively (Table 2.1). The brains were removed and the slices incubated as described in the legend. Using values from ten routine experiments (not identical with those of Table 2.1) an attempt was made to correlate the delays in incubation in the present experiments with the swelling, and with the Na^+ and K^+ values measured after incubation under identical conditions (standard media, 30 min preincubation and 60 min incubation). However, none of the values correlated with the delays. The reasons for the discrepancy between the present experiments and those of Varon & McIlwain and Bachelard et al. might be the following: First, the delays in the present experiments varied less (removal $\pm$ 0.8 min, incubation $\pm$ 1.7 min, S.D. of 57 slices) and, due to the heterogeneity of the material, the overall error of the measurements was greater than in the material of the authors mentioned. Such characteristics of the variables would diminish the statistical significance of the regression lines

of the present experiments. Secondly, the incubation times were longer than those of Varon & McIlwain (60 min) and Bachelard et al. (30 min). Differences in the condition of the slices before incubation would tend to even out due to uncontrolled variations during incubation. Thus in the analysis of variance on the results (Table 4.3 - 4.5) it was often found that the variations between slices in two incubation beakers with the same media was greater than that between the first and second slice in each beaker (these variations are not shown in Table 4.3 - 4.5). Thirdly, in the present experiments, the brains were kept in a cold and moist atmosphere immediately after removal. This has been shown to preserve resting potentials of cerebral slices for several hours (Hillman & McIlwain, 1961) and therefore, implicitly, the K^+ and Na^+ of the slices. Fourthly, the animals were anaesthetized before killing by neck fracture in present experiments, whereas they were stunned by a blow on the neck and exsanguinated in the experiments of Varon & McIlwain. However, Bachelard et al. showed that anaesthesia did not influence the results.

ii) The cutting of the slices

With some experience, slices can be cut from the organs free hand, as was originally done (Warburg, 1923). However, this is not easy with a soft tissue like the brain. Various methods have been introduced in order to make the process easier and faster and to produce uniform slices with as little damage as possible. The McIlwain-Buddle tissue chopper, the blade and recessed guide, the bow cutter and the Stadie-Riggs microtome have been described in detail (McIlwain & Rodnight, 1962; Elliott, 1969). A relatively recent method, using a motor-driven fine nylon thread to cut the slices (Franck et al., 1968) was used in the present experiments (Fig. 2.1). The functional state of slices cut by different methods under comparable conditions does not seem to have been examined. However, a) differences between slices with one

and two cut surfaces, and b) differences between the outer and inner layers of slices have been seen in the use of all methods in which these things have been examined.

a) Slices with one and two cut surfaces were seen to differ with respect to inulin space and swelling after 1 h incubation by workers using the blade and guide method (Varon & McIlwain, 1961). Use of the same method, but cutting the slices without removal of the brain, 'cutting in situ' (Hillman et al., 1963), yielded slices in a more satisfactory state. However, the same difference as above was measured in the inulin space between the 'first' and the 'second' slice, as well as a lower concentration of K^+ and a higher concentration of Na^+ in the second slice (Keesey et al., 1965). Franck et al. (1968), in a paper describing their new method, reported significant differences in the swelling, the K^+ and the Na^+ between the first and second slice. Hillman et al. (1963) demonstrated that the cell population of the first and the second slice are different. However, this is unlikely to explain the differences in functional activity, mentioned above. Firstly, the concentration of representative enzymes, and hence the metabolism, was found to be independent of the structural elements in the upper cortical layers (Robins et al., 1956). Secondly, slices cut from areas of the brain with cells more different than those from two cortical layers showed no distinct difference in the K^+ and Na^+ or tissue water (Hertz et al., 1970).

b) Various methods have been used to assess differences between the outer and inner layers of the slices. Pappius et al. (1962) using the Stadie-Riggs microtome estimated cellular damage microscopically by means of fluorescence labelled protein and inulin space, and interpreted their findings as evidence of extensive damage along the cut edges (see also section 3iii) of this chapter). Sershen & Lajtha (1974) after incubation of 0.42 mm slices in standard media cut them in sections of 0.050 mm parallel to the surface, and measured the contents of various substances from surface to centre.

They found the water content, as measured by the urea space, uniform throughout the slice. The inulin space was greater at the surface, indicating penetration of injured cells. The ATP concentration was highest at the centre, whereas both the Na^+ and K^+ were found to be higher at the surface than the centre. No explanation was offered for the latter finding, which the authors also found surprising. In cortical 'first' slices, 0.6 - 0.7 mm in thickness, the concentrations of K^+ and ATP at the intact surface were somewhat higher than at the cut surface, indicating a 'healthier' state of the former part of the slice; otherwise the findings were the same as above. The thin and thick slices were cut, using the McIlwain-Buddle Chopper (two cut surfaces) and the blade and guide, respectively. This shows again that the cutting itself is more important than the method of cutting (about the thickness, see iii)). Cohen (1974b) cut slices the thickness of which was increased stepwise from 0.16 mm to 0.69 by means of the McIlwain-Buddle Chopper. In these experiments the slices were incubated for varying periods in the presence of inulin and sucrose, whose spaces were measured as well as the total water content. (The experiments were different from those assessing the adhering fluid (Cohen, 1974a) mentioned in section 3iii) of this chapter). It was found that cutting affected the tissue to considerable depth. There was some experimental support for a 'sandwich' model of the slice; slices thicker than 0.3 - 0.35 mm, cut on both sides, appeared to consist of two exterior layers, 0.15 - 0.17 mm thick, of altered tissue with a central core of relatively intact tissue. Under all experimental conditions there were transition zones between 'exterior' tissue and 'core' tissue. At 37°C the differences between the water content, the sucrose space and the inulin space of the two zones were much less than predicted by the model. Furthermore, the tendency to even out the difference between the zones was clearly dependent on the incubation time in the case of inulin.

It is not surprising that a static model like the 'sandwich' slice gives the best fit at 0°C and short incubation times. The model assumes adequate oxygenation of the innermost layers of the thickest slices used in the experiment. This is most likely to be true under the latter conditions. In the paper on swelling mentioned earlier (Møller et al. 1974) in which the apparatus of Franck et al. (1968) was used, the authors found evidence of swelling at the periphery. This was also true for the area just below the uncut surface, although it was less pronounced. The findings, which were confirmed by determination of the water content in 0.05 mm sections parallel to the surface of the slices, demonstrate that factors other than the cutting procedure can be responsible for the superficial damage. Exposure to the laboratory atmosphere during removal, possible toxicity of the high oxygen tension at the surface (Elliott, 1969, p. 109) and possible mechanical damage due to the vigorous oxygenation used by the authors would be factors common to both surfaces of their slices.

Collectively, the evidence from the literature indicates that, irrespective of the method used, the unavoidable cutting of the slices affects the tissue considerably, but that other factors must also be responsible for some of the superficial damage.

As a consequence of the differences between the slices from two cortical layers demonstrated by several authors, including Franck et al. (1968), only slices with one cut surface were used in the present experiments.

iii) The thickness of the slices

The permissible thickness of the tissue slice is determined, on the one hand, by the distance of diffusion of oxygen and substrates to the inner layers of the tissue and, on the other hand, by the proportion of the tissue that is damaged in the cutting procedure. In his introduction of the tissue

slice as a preparation, Warburg (1923) calculated the limiting thickness at which the innermost layer of the slice is just not oxygenated. Warburg's equation states that the limiting thickness, d' (cm), equals $(8 C_O D/A)^{1/2}$, in which C_O = the oxygen concentration (atmospheres), D = Krogh's diffusion coefficient for O_2 in tissue at $38^\circ C$ in cm^3 per cm^2 , per min, when the pressure difference is 1 atmosphere per cm ($= 1.4 \times 10^{-5} cm^2 min^{-1} atm^{-1}$), and A = the oxygen consumption in cm^3 per cm^3 of tissue per min ($= 5 \times 10^{-2} min^{-1}$ for liver tissue). Warburg calculated a limiting thickness for liver slices, gassed with 100% oxygen, of 0.047 cm. For slices gassed with atmospheric air the limiting thickness was 0.022 cm.

On the basis of Warburg's equation, McIlwain & Rodnight (1962, p. 109) recommended a maximal thickness of 0.4 mm for cerebral slices. Using their value for the respiratory rate of $150 \mu moles O_2/g/h$ ($= 150 \times 22.4 \times 10^{-3}/60 = 5.6 \times 10^{-2} min^{-1}$), a value for d' of 0.045 cm is found. Also, the above figure for the respiratory rate is only seen during stimulation of the tissue, which was not used in the present experiments (McIlwain & Bachelard, 1971, Table 4.1; Hertz, 1973, Table VI, 1). Longmuir & Bourke (1959), measured the critical oxygen concentration of tissue slices and found that it was largely independent of the thickness. These results, which are contrary to the predictions of Warburg's equation and which were found in soft tissue like the liver as well as hard tissue like the kidney and heart, question the use of Krogh's figure. The authors used slices of a range of thicknesses in the ratio of 1:5, but did not specify the actual thickness of their slices. Clearly, there must be a limiting thickness, as is implied by the presence of a capillary system. Warburg (1923) referred to experimental evidence for his equation (Minami, 1923). The three slices with thicknesses greater than the limiting value were 0.5, 0.95 and 1.24 mm

(1.c. Table III), and in spite of the small number, their respiratory rates are significantly different from those of the control values ($p < 0.02$, not tested by the author). However, the respiratory rate, by which the limiting thickness was estimated in this experiment, and the critical oxygen concentration in that of Longmuir & Bourke (1959) reflects the proportion of functioning cells in the slices. Therefore, if Krogh's figure underestimates the rate of diffusion, the apparent discrepancy of the two experiments may be a question of the absolute thickness of their slices and the method of preparation.

Practical experience with the use of cerebral slices 0.5 mm in thickness shows that their functional activity is satisfactory. The oxygen uptake per unit weight is not increased by the use of thinner slices (Elliott, 1969) and the concentration of K^+ of the slices indicates optimal conditions (Arnfred et al., 1970; Lund-Andersen & Hertz, 1970). Thick slices are less easily torn, and the very thickness may be a prerequisite for the vigorous oxygenation used by the latter authors.

In the present experiments 0.5 mm thick slices were used.

iv) The transfer of the slices after cutting to incubation
(Fig. 2.1 & 2.2)

As mentioned above i) the brains were placed on moist filter paper in a Petri dish after removal. The cutting (Franck et al., 1968) made it unnecessary to handle the slices in aqueous fluids (McIlwain & Rodnight, 1962, p. 110 ff.), as this has been found to cause increased swelling (Varon & McIlwain, 1961; see also i)). After the cutting, the slice was picked up by means of a bent rider, weighed to the nearest milligram on a torsion balance (White Electrical Instr. Co.) and floated in the incubation beaker. This whole procedure took about 3 min (Table 2.1, difference in delay between the 1st and the 2nd slice), during which period the slices were exposed to the laboratory atmosphere. Elliott (1969) recom-

mends the cutting of the slices in a humid chamber, but the effect of this does not seem to have been assessed.

1B) THE EFFECTS OF INCUBATION

i) The nominal composition of the medium

The slices were preincubated for 30 min in a Krebs-Ringer bicarbonate glucose saline at 37°C, aerated vigorously with a water saturated mixture of 95% O₂:5% CO₂ (or 95% N₂ : 5% CO₂, when anaerobic conditions were used). The incubating medium referred to later as 'standard' had the composition shown (Table 2.2). The preincubation had the aim of allowing the slices to regain K⁺ and extrude Na⁺ after the initial shifts (Pappius & Elliott, 1956b; Bachelard *et al.*, 1962; Franck *et al.*, 1968) and was obligatory, except in a few cases which will be mentioned as they arise. Deviations from the composition of the standard medium often took place and will also be mentioned in each particular case. However, these intentional modifications of the medium were part of an experimental design. Unintentional modification of the medium might easily confound the results of a design unless they were controlled or at least known. Disregarding gross errors of concentrations of the solutions, the use of decomposed solutions etc., there remain some possibilities of unintentional modifications of the medium a) evaporation, b) changes of the pH, c) the leakage of substances from the slices.

a) Evaporation: Vigorous aeration of the medium is essential, as will be mentioned later. However, at 37°C the vapour pressure is not negligible (Table 2.2), corresponding to 6% of the gas mixture that leaves the medium. Therefore, solutes in the medium may be concentrated to an unknown degree depending on the flow rate and the volume of the medium.

In the present arrangement (Fig. 2.3), the gas was saturated at 37°C in a wash bottle. Manifolds and tubes that could not be kept under water were either insulated or heated by a lamp

(not shown). Evaporation was estimated by simultaneous measurement of the Na^+ and K^+ of the media, and by weighing the media, before and after incubation (Table 2.3A). The errors of these measurements are not the same. Therefore, the identical results reflect not only the accuracy (nearness to the true value) of the estimate, but also the accuracy of the Na^+ and K^+ measurements (see section 2). The evaporation was also controlled by difference of weight in routine experiments of various length and corresponded well with estimates of the Li^+ concentration in the media (Table 2.3B) and the previous estimates (Table 2.3A). The advantage of having a medium of relatively high volume is obvious. Under these circumstances the effects of evaporation could be disregarded.

b) Changes of the pH: The standard medium (Table 2.2) uses the bicarbonate- CO_2 system as its main buffer. It is recommended because of its similarity to the plasma and cerebrospinal fluid (McIlwain & Rodnight, 1962, p. 134 - 135). However, the buffer capacity of the system is less than is generally considered optimal for a buffer. This is because of the high ratio between HCO_3^- and CO_2 . Using a value for the solubility of CO_2 and 20°C of 0.878 and a mean atmospheric pressure of 740 mm Hg, the $\text{CO}_2\%$ can be converted to $\% \text{CO}_2 \times (740 \times 0.878 \times 10^3 / (760 \times 22.4 \times 10^2)) \text{ mM} = \% \text{CO}_2 \times 0.38 \text{ mM}$, or 1.9 mM at 5% (Umbreit et al., 1957, p. 21 - 23). By means of the Henderson-Hasselbalch equation it can then be calculated that the fairly modest changes of CO_2 in the gas mixture of 5.5% to 4.5% ($\text{HCO}_3^- = 26 \text{ mM}$) or of bicarbonate in the medium from 23 mM to 29 mM ($\text{CO}_2 = 5\%$) would increase the pH of the medium by 0.1 unit. Representative measurements of the pH in the present experiments are shown (Table 2.4). The small standard deviation of the repeated measurements indicates the uniformity of the media with respect to CO_2 and bicarbonate. It has also been recommended to 'measure roughly' the pH of the media at the end of the incubation because lactic acid may lower the pH (McIlwain & Rodnight,

1962, p. 138). However, bicarbonate is an intermediate ion of a polyprotic acid whose pH is almost independent of its concentration. Consequently, the measurements after 1 h and 3 h (Table 2.4) only show that some bicarbonate still remained and that CO_2 was diffusing away. It was not practicable to measure the pH immediately after the incubation, therefore these measurements were soon abandoned.

The pK' of 6.4 at 20°C used in the calculations of Umbreit et al. (Table 7) would predict a pH of 7.5 instead of the 7.2 found in the present experiments (Table 2.4). The pH meter (EIL 38a) was checked with primary standards; there was no reason to suspect systematic errors in the measurements. Keesey et al. (1965), using $\text{HCO}_3 = 26 \text{ mM}$, found the pH of 7.65 at 37°C shown in the chart of Umbreit et al. (p. 25). Other authors using the same concentrations reported values between 7.2 and 7.4 but the temperatures were not stated (Hillman & McIlwain, 1961; Franck et al., 1968). The pH increases about 0.1 from 20 - 37°C (Umbreit et al., p. 20). The pH of the cerebral slices is lower than that of the medium (Hertz et al., 1970) and changes less than that of the medium (Elliott & Birmingham, 1949). This may be the reason why changes of pH from 6.1 to 8.4 caused relatively small changes in the respiratory rate of cerebral slices (69% - 116%, respectively, 100% at pH 7.2, Cohen, 1973). Considering the fairly low buffer capacity of the widely used bicarbonate- CO_2 system, the broad range of pH values reported, the different intracellular pH and the respiration of a large proportion of the cells in spite of biologically extreme pH values, it may be safely concluded that small changes of the pH during incubation are of little consequence for the functional activity of the slices. Control of the pH may then serve as a check against gross errors in the composition of the medium before incubation, as it did in the present experiments.

c) The leakage of substances from the slices: There was a significantly higher concentration of K^+ in the media bubbled with N_2 than in the oxygenated media (Table 2.5). The difference may well represent a loss of K^+ from the incubated slices (Table 2.11a) to the medium. Using the average incubated wt. of the four slices, this loss was found to be of the same magnitude, namely $82.8 \text{ mg } (56.2 - 15.4) \times 10^{-3} \mu\text{mol mg}^{-1} = 3.4 \mu\text{mol per } 10 \text{ ml of the medium or } 0.3 \text{ mmol/l}$. The difference measured in the medium represented 2 - 3 units on the scale of the flame photometer. The almost equimolar uptake of Na^+ from the medium of $82.7 \text{ mg } (150.7 - 104.6) \times 10^{-3} \mu\text{mol mg}^{-1} = 3.8 \mu\text{mol/10 ml}$ would not be recorded because of the twelvefold higher dilution. Changes of the K^+ and Na^+ of the media due to the incubation of fresh slices would be of the same magnitude as above. Such changes could be disregarded for most practical purposes because of the high volume of the medium (10 ml). However, in some experiments, nominal concentrations of K^+ of 0 and 1 mM were compared (Fig. 4.2, Table 4.3) in which the contribution from the slices would be meaningful to discuss. Similar reasoning would apply to the efflux experiments with Li^+ and are discussed in Chapter Six.

ii) Changes interfering with the metabolism of slices

The importance of adequate oxygenation has been emphasized since the tissue slice was introduced in experimental biology (Warburg, 1923). Franck et al. (1968) found that complete anoxia led to concentrations in the slices near those of the medium. Optimal metabolic conditions, judged by the K^+ , Na^+ and swelling of the slices, were obtained when the slices were agitated continuously by the oxygen, whereas restriction of movement led to changes suggestive of partial anoxia. Similar findings on the effect of agitation of slices were made by other authors (Hertz, 1968; Arnfred et al., 1970). The effect of total anoxia was also observed in the present experiments (Table 2.6b).

However, the concentrations of K^+ observed in the present experiments in a standard medium (Table 2.6a) would seem to indicate less favourable conditions than those of the authors mentioned above, who obtained values for the K^+ in slices of 70-75 mmol/kg incubated wt. In the absence of Li^+ , values near 65 mmol/kg incubated wt. were found (Wraae et al. 1976), but the statistical significance of this finding is doubtful (see section 4i) of this chapter). The practical arrangement of Arnfred et al. (1970, Fig. 1) using a narrow tube that does not allow the slice to escape from the gas stream is superior to the one used in the present experiments (Fig. 2.3) with respect to the continuous movement of the slices during incubation.

The concentrations of glucose in the medium necessary to maintain the metabolism of cerebral slices were studied early (McIlwain, 1953); thus the concentration of 10 mM the standard medium provides an excess for all possible circumstances of incubation. In the present experiments, the omission of glucose from the medium caused a shift in the K^+ and Na^+ of the slices similar to that observed during anoxia, although less pronounced (Table 2.6c).

Omission of Ca^{2+} from the medium stimulates both the oxygen consumption and glycolysis of cerebral slices (Bourke & Tower, 1966b). The concentration of Ca^{2+} in the medium also influences the K^+ , Na^+ and the swelling of slices (Joanny & Hillman, 1964; Keesey et al., 1965). This effect was not statistically significant in all of the present experiments (Table 2.6d), whereas it was clearly demonstrated in some, (Fig. 4.4). The reasons for this discrepancy may be the incubation time which has been found to influence the results (Joanny & Hillman, 1964).

The influence on the Li^+ in the slices was highly significant (Table 2.6, column 2). As these findings were original, they were later subjected to further control experiments (Table 2.10).

iii) The duration of the incubation

Franck et al. (1968) found that under optimal conditions, slices could be incubated for several hours without any signs of decreased viability, whereas insufficient oxygenation caused a continuous loss of K^+ and an increase in swelling. In the present experiments, the only sign of deterioration was a slight increase in the swelling between 30 min and 120 min incubation (Table 2.7). The main changes in the K^+ of the slices and in the swelling take place within 30 min (Pappius & Elliott, 1956ab; Varon & McIlwain, 1961; Bachelard et al., 1962; Bourke & Tower, 1966ab; Franck et al., 1968; Hillman et al., 1974). Therefore, these changes would not be observed in the present experiments because of the preceding period of preincubation. The results shown (Table 2.7) were taken from several experiments, and experimental scatter may have hidden true differences. In the experiments of Chapter Six (Table 6.1) there were statistically significant differences between the concentrations of K^+ in slices incubated for 60 min and 120 min.

iv) Control experiment with homogenates

The ratio of concentration of Li^+ between the slices and the media was higher than one under standard conditions of incubation. Using the values from Table 2.6a, a concentration ratio of 1.34 is found. The possibility was considered that this ratio might be due to an unspecific affinity for components of the tissue. Assuming the surplus Li^+ to be associated exclusively with the solids, and assuming these to be 0.2 of the total weight of the tissue, then the ratio of Li^+ between the solids and the water of the tissue would be $(1.34 - 0.8)/0.2 = 2.70$, (see Chapter Three, METHODS, for this type of calculation). In the experiment (Table 2.8), the added volume of the medium would make the solids approx. 0.05 of the total weight of the homogenate. All other things equal, the amount of Li^+ in the homogenate associated with the solids would now be $2.70 \times 0.05 = 0.14$. Therefore, the

recovery of Li^+ in the supernatant would be $(1 - 0.14) \times 100 = 86\%$ of a sample having the same amount of Li^+ and the same total weight, but no tissue. On the other hand, if the solids were inert towards Li^+ , the supernatant should show a recovery of approx. 105%, because there was a surplus of Li^+ - containing fluid in the control solution corresponding to the weight of the solids. In practice, the control value was higher than the expected 100%. This could either be due to a systematic error in the volume of the pipette, or to the Krebs-Ringer solution interfering with the measurements of Li^+ , as was in fact found later (see section 2 iii)). However, the recoveries were not significantly different, and it can be calculated that differences of nearly 10% would have to be found in order to show statistical difference ($P < 0.05$) in the presence of the variation observed and the number of samples used. Such a recovery would require almost total association of the surplus Li^+ with the solids according to the previous calculation. Therefore, the experiment was not conclusive. It was not pursued further because a more suitable control was found.

v) The use of fixed slices

Fixatives are used to preserve the structure of the tissue for microscopical examination. In the present experiments, fixed cerebral slices served as a model that was likely to have the same structure as an unfixed slice, but no metabolism. Formalin was chosen as a well-known and generally accepted fixative. Chronologically, the effects of Ca^{2+} on the Li^+ uptake were the first that were found. Therefore, the effects of this ion were examined most thoroughly in control experiments, in order that artifacts of the measurements might be excluded. In these control experiments fixed slices were used.

In the comparison between fixed and unfixed slices, the

swelling is the easiest quantitative criterion to use. As stated earlier, swelling can be caused by many agents and the phenomenon is incompletely understood. However, gross dissimilarity of swelling between unfixed and fixed slices would make the latter preparation unsuited as a structural model of an unfixed slice. The effects on swelling of formalin and formol-saline were examined. (Table 2.9). The solutions were made from conc. formalin (40% w/v) by tenfold dilution with either distilled water or saline. Accordingly, they were 4% w/v and not 4% v/v, as was stated earlier (Wraae et al., 1976, p. 836). The swelling caused by 'pure' formaline was pronounced and took place not only during fixation, but also during incubation (Table 2.9, ax and ay). Slices fixed in formol-saline swelled approx. 25% after fixation and incubation. There was a small, but significant decrease of the swelling during incubation (Table 2.9, cx and cy). In 8 slices, the swelling was also measured separately after both the fixation and the rinse, but no significant difference was found (not shown). The most likely explanation for the observed decrease in swelling after incubation is evaporation of adhering fluid owing to handling in the laboratory atmosphere of the 37°C warm slice. The fresh weights of the slices in group (a) and (c) were significantly different. However, this could not explain the difference of swelling (Table 2.9, al and bl, ay and by). As a result of the experiments (Table 2.9), slices fixed in formalin were found unsuitable as controls. Slices fixed in formol-saline swelled to approximately the same extent as incubated slices and were therefore used.

The effects of glucose and Ca^{2+} on the Li^+ in fixed and unfixed slices were examined (Table 2.10). The statistical analysis showed some overlap of the results, but the following conclusions can be drawn: In the unfixed slices, the concentration of Li^+ was higher than that of the medium under standard conditions, which included the presence of glucose

and Ca^{2+} . The absence of glucose lowered the concentration of Li^+ in the slices, whereas absence of Ca^{2+} increased the concentration of the Li^+ , but only when glucose was present in the medium (Table 2.10c). All the concentrations of the Li^+ of the slices associated with these variations of the medium were significantly different, not only from the concentrations of unfixed controls, but also from those of the fixed slices. No significant changes were seen in the Li^+ of fixed slices subjected to the same variations of the medium. The concentrations of Li^+ in the fixed slices will be commented on at the end of this section.

The effects of variations of the medium on the K^+ and Na^+ in unfixed slices (Table 2.11a and b) were similar to the results presented earlier (Table 2.6b and c). The K^+ and Na^+ in the slices were not measured in the absence of Ca^{2+} only, in these experiments, but are shown elsewhere (Table 2.6d; Fig. 4.4). The simultaneous absence of Ca^{2+} and glucose had an effect similar to that of anoxia (Table 2.11c). All the K^+ and Na^+ values of the experimentally tested slices of column 2 were significantly different from the control values of column 1 as well as those of the fixed slices. None of the values in the fixed slices changed with the experimental conditions. The K^+ concentrations of the fixed slices were similar to those of the media (Table 2.5), whereas the Na^+ values were generally higher. This will be discussed at the end of this section.

The swelling (Table 2.12) of the unfixed slices increased in the presence of all the changes of the medium that were associated with changes in the Li^+ (Table 2.10), K^+ and Na^+ (Table 2.11) of these slices. The increase of the swelling in the absence of Ca^{2+} was not statistically significant in this experiment, but became so when a greater number of slices were used (Table 4.4). In the fixed slices, there was no statistical difference of swelling, neither within experiments (Table 2.12, column 3 and 4) nor from one experiment to

another (not shown). The mean value was approx. 25% which was intermediate between the mean swelling under standard conditions (approx. 15%) and under variations in the medium (approx. 40%).

In the experiments (Table 2.10 - 2.12), conditions of incubation that are known to cause changes in metabolism were associated with substantial changes in the K^+ , Na^+ and swelling of unfixed slices, whereas none were seen in the fixed slices. The uneven distribution of Li^+ in the unfixed slices between the tissue and the medium, especially in the absence of Ca^{2+} might then be interpreted as being dependent on metabolism. However, the ionic and other changes in the slices caused by a change in metabolism might also change the Li^+ measurements. Consequently, the absence of ionic and other changes in the fixed slices might be the very reason for the stability of the Li^+ signal. The second objection to the use of fixed slices as controls was the concentrations of Li^+ and Na^+ , which were often higher than those of the media. This could be due to the measurements, which were carried out early on and were not as well standardized as they were in later experiments. However, this should apply equally well to the unfixed slices; furthermore, the Li^+ signals of standards were identical in the presence of fixed and unfixed cerebral tissue; finally, the later recovery experiments (Table 2.15) showed no interference with the Li^+ measurements in spite of great changes in the experimental conditions. Therefore, the low concentrations of Li^+ in the unfixed slices in the absence of metabolism, and the high concentrations in the fixed slices under all experimental conditions, must be true values. This removes the first objection to the use of fixed slices as controls. The second objection is less easy to eliminate. A possible explanation for the higher Li^+ and Na^+ values of the fixed slices might be that the precipitation of the proteins during fixation would alter the affinity to ions of the

fixed tissue; one would then have to assume a different affinity for K^+ . However, the structural changes of the tissue after anoxia are extensive (Møller et al., 1974), so that one might just as well expect an altered affinity to ions after anoxia. It could also be postulated that the fixed slice represents the true non-metabolizing tissue and that, in the absence of metabolism, the unfixed tissue alters its affinity to ions. One would then have to assume that non-metabolizing tissue has a high affinity for Na^+ and Li^+ and none for K^+ , but due to swelling etc. the affinity of unfixed tissue to Na^+ increases too little, the affinity to Li^+ decreases too much, and that of K^+ remains approx. the same. This does not make the assumptions simpler.

None of the models of non-metabolizing tissues were quite satisfactory and the discrepancies between them could not be clarified without further experiments which would be beyond the scope of the present thesis. Altogether, the fixed slices seemed less suited as inert controls than was originally thought, and it was found that recovery experiments would give more valuable information about the validity of the results.

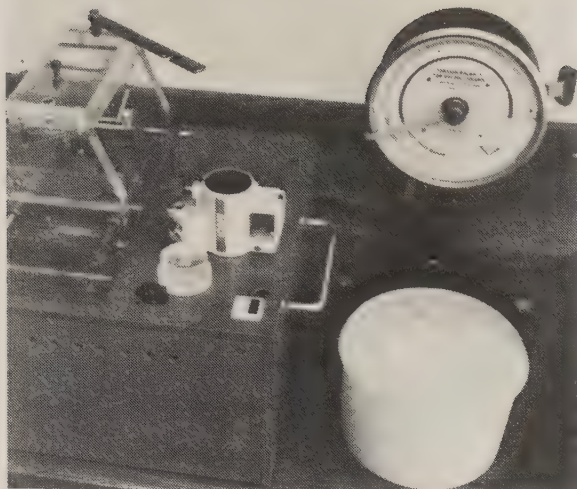


FIG. 2.1

The arrangement for
the cutting, storage
and weighing of slices

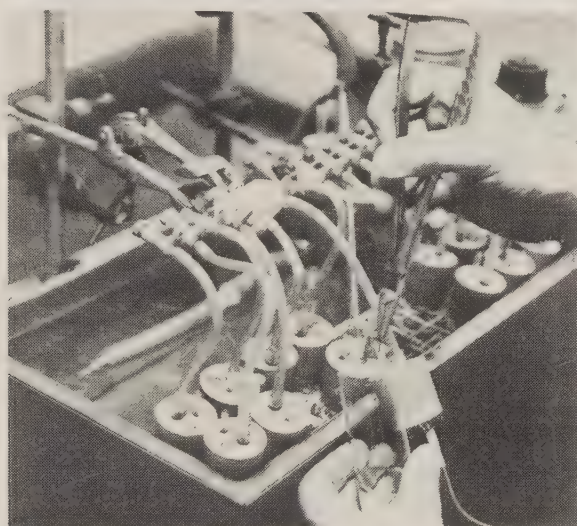


FIG. 2.2

The transfer of the slices
for the incubation

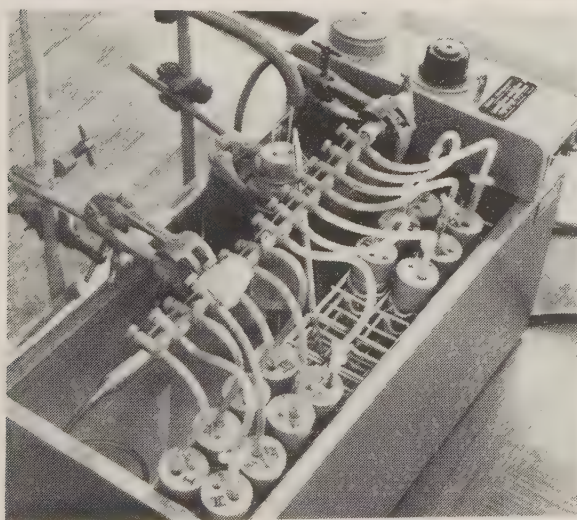


FIG. 2.3

The incubation of the slices,
Manifolds and tubes above
water were either insulated
or heated by a lamp (not
shown)

TABLE 2.1 THE DELAY IN THE INCUBATION OF CEREBRAL SLICES

	removal of brain (min)	incubation of 1. slice (min)	incubation of 2. slice (min) *
mean $\pm$ S.D.	2.65 $\pm$ 1.17	6.88 $\pm$ 2.73	9.78 $\pm$ 2.90

- 1) Rats were killed by neck fracture - after light ether anaesthesia. The brain was removed (McIlwain & Rodnight, 1962, p. 1-3) and placed on moist filter paper in a Petri dish on ice.
- 2) One cerebral slice was cut from the lateral surface of one of the hemispheres (Franck *et al.*, 1968) and weighed before incubation on a torsion balance. The arrangement is shown (Fig. 2.1).
- 3) A second slice was cut from the other hemisphere and treated like the first slice. Two experimental workers carried out 1), and 2) - 3), respectively. The duration of the delays presented was estimated from the time when the animals were killed and was based on 100 slices in 11 routine experiments, from 4 periods during which the workers had various degrees of experience.

* Note that this slice has one intact surface also.

TABLE 2.2 THE COMPOSITION OF THE STANDARD MEDIUM

cations	Na ⁺	K ⁺	Ca ²⁺	Mg ²⁺
(mM)	150	6.2	2.6	1.3
anions	Cl ⁻	HCO ₃ ⁻	H ₂ PO ₄ ⁻	SO ₄ ²⁻
(mM)	134	26	1.2	1.3
substrate (mM)	glucose : 10			
gasses (mmHg)	O ₂ /N ₂ : 677	CO ₂ : 36	H ₂ O : 47	

In this table, and all other tables, the following notations are used for the concentrations of ions and most other solutes: Nominal concentrations: mM; measured concentrations: mmol/l or mmol/kg incubated wt. The media were made up according to Umbreit *et al.* (1957, p. 149).

TABLE 2.3

ESTIMATION OF EVAPORATION FROM THE MEDIUM

A. Simultaneous measurement of weight, Na^+ and K^+ before and after incubation.

	decrease in weight (%)	increase in Na^+ (%)	increase in K^+ (%)
mean	3.96	3.45	5.0
S.D.	0.78	1.53	2.21
no. of media (experiments)	12 (2)	12 (2)	12 (2)

Cerebral slices were incubated for 1 h in a standard medium of 10 ml (100%). The incubation beakers were weighed before incubation. After incubation they were sealed with parafilm and allowed to cool. Condensed water was wiped off on the outside and inside and the beakers were reweighed. The concentrations of Na^+ and K^+ in the incubated media were measured by flame photometry using an unincubated medium as a standard (100%). The analysis of variance showed no significant difference in the three ways of estimating the evaporation from the medium.

B. Comparison of weight and Li^+ in several media.

	1) decrease in weight (mg)	2) concn of Li^+ (mmol/l)
mean $\pm$ S.D.	362 $\pm$ 127	1.537 $\pm$ 0.058
no. of samples (experiments)	146 (20)	219 (25)

The decrease in weight was estimated as above in media, 5 ml or 10 ml, subjected to incubations of various lengths. The concentration of Li^+ in the medium of 10 ml was estimated in 25 consecutive experiments in which the Li^+ concentration was made up to be 1.5 mmol/l. The experiments in 1) and 2) were identical in only 11 cases.

TABLE 2.4 CONTROL OF THE pH OF THE MEDIUM

	1) before incubation	2) after incubation	
		a) approx 1 h	b) approx 3 h
mean $\pm$ S.D.	7.22 $\pm$ 0.035	7.70 $\pm$ 0.132	7.98 $\pm$ 0.027
no. of samples (experiments)	40 (25)	40 (6)	6 (1)

The pH was measured at room temperature by means of an EIL 38a pH meter.

- 1) routinely after preparation and aeration of the Krebs-Ringer solutions with 95% O₂ : 5% CO₂ or 95% N₂ : 5% CO₂ before incubation.
- 2) in some of the samples after incubation.

TABLE 2.5 THE EFFECTS OF O₂ AND N₂ ON Na⁺ AND K⁺ IN THE MEDIUM

95% O ₂ : 5% CO ₂	147.7 $\pm$ 3.6	6.83 $\pm$ 0.05
95% N ₂ : 5% CO ₂	154.3 $\pm$ 9.7	7.08 $\pm$ 0.05
P value of difference	n.s.	< 0.001

Media from the experiment (Table 2.11 a) were used. They were diluted with distilled water by 300 times before the measurement of Na⁺ and 25 times before the measurement of K⁺ by flame photometry. Each value presented is the mean $\pm$ S.D. of four samples. Student's t-test was used to evaluate the significance of the differences.

TABLE 2.6 EFFECT OF VARIATIONS IN THE MEDIUM ON Li^+ ,
 K^+ AND Na^+ IN INCUBATED CEREBRAL SLICES

Composition of the medium	Li^+ (mmol/l or kg) medium	slices	K^+ (mmol/kg) slices	Na^+ (mmol/kg) slices
a) standard medium	± 1.556 $\pm 0.041(77)$	± 2.091 $\pm 0.161(37)$	± 56.2 $\pm 8.8(37)$	± 101.0 $\pm 7.7(37)$
S.E.M.	0.0304(9) (n.s.)	0.0863(9) $P < 0.001$	5.10(9) $P < 0.001$	3.53(9) $P < 0.001$
b) no oxygen	± 1.570 $\pm 0.025(8)$	± 1.510 $\pm 0.058(8)$	± 9.28 $\pm 0.64(8)$	± 158.5 $\pm 2.5(8)$
c) no glucose	± 1.532 $\pm 0.052(8)$	± 1.731 $\pm 0.094(6)$ $P < 0.01$	± 17.82 $\pm 2.91(6)$ $P < 0.001$	± 153.4 $\pm 4.7(6)$ $P < 0.001$
glucose (10 mM)		2.108(2)	57.69(2)	114.9(2)
d) no Ca^{2+}	± 1.572 $\pm 0.034(10)$	± 2.947 $\pm 0.090(4)$ $P < 0.001$	± 47.27 $\pm 2.37(4)$ (n.s.)	± 110.2 $\pm 2.9(4)$ (n.s.)
Ca^{2+} (2.6 mM)		± 2.084 $\pm 0.223(4)$	± 59.25 $\pm 9.90(4)$	± 105.3 $\pm 13.0(4)$

Cerebral slices were incubated for 1 h. in a medium also containing Li^+ 1.5 mmol/l. The values presented are the means $\pm$ S.D. of the number of samples shown in brackets. The figures in a) are the joint values from 9 separate experiments; as the analysis of variance showed a significant variation between the 9 experiments the statistical comparison between results in a) and b) was based on the variance between the experiments, and the number of experiments; the resulting standard errors are also shown. Student's t-test was used to compare the means between which the p values are placed.

TABLE 2.7

THE EFFECT OF INCUBATION TIME ON THE SWELLING
AND K^+ OF CEREBRAL SLICES

	swelling (% of fresh wt.)	K^+ concn (mmol/kg)
Intercept (a)	14.1	61.5
regression line (b) (min^{-1})	0.050	- 0.043
correlation coefficient	0.28 (p < 0.05)	0.14 (n.s.)
no. of slices (experiments)	59 (6)	59 (6)

Cerebral slices were preincubated for approx. 30 min in a standard Krebs-Ringer solution. They were then incubated, without transfer, in the same solution, also containing Li^+ 1.5 mM, for periods of 12 min to 90 min ($47 \text{ min} \pm 24$, mean $\pm$ S.D.). The slices were weighed before and after preincubation + incubation (mean fresh wt $\pm$ S.D. = $62.3 \text{ mg} \pm 11.1$). The swelling was calculated from the weight increase, and the K^+ concentrations were referred to the incubated weights. The intercept (a) in the regression equation, $y = a + bx$ indicates the swelling or K^+ concentration at the end of the preincubation.

TABLE 2.8

THE AFFINITY OF Li^+ FOR TISSUE HOMOGENATES

	(a) kidney (%)	(b) liver (%)	(c) brain (%)	(d) control (%)
mean value	108.8	111.5	100.3	107.0
S.D. (no. of samples)	5.2 (5)	3.8 (5)	8.5 (3)	7.2 (6)

Weighed samples of kidney, liver and whole brain from 3 rats were homogenised by hand in known volumes (approx. 4) of a standard Krebs-Ringer medium. One to six aliquots (5 μl) of a concentrated Li^+ solution were added. The homogenates were aerated with a mixture of 95% O_2 : 5% CO_2 for 10-15 minutes. They were then separated by centrifugation and Li^+ was measured in the supernatants against a corresponding standard in distilled water (100%). Tissue blanks were included. The figures in column (d) represent samples of the same total weight but without tissue. The analysis of variance showed no significant difference in the recoveries of Li^+ .

TABLE 2.9 THE EFFECT OF THE FIXATIVE, FIXATION AND INCUBATION
ON THE SWELLING OF FORMALIN FIXED SLICES

fixative	formalin	for-sal	for-sal
groups	(a)	(b)	(c)
no. of slices	16	14	16
1) fresh wt. (mg)	63.6 $\pm$ 7.6	61.3 $\pm$ 6.3 ***	50.1 $\pm$ 8.7
2) swelling (% of 1)			
x) after fixation and rinse	75.4 $\pm$ 10.0 ***	***	30.3 $\pm$ 5.2 **
y) after incubation	90.4 $\pm$ 8.9 ***	25.9 $\pm$ 4.3	27.7 $\pm$ 4.1

Preparation:

Cerebral slices were cut and weighed. They were kept overnight in a 4% (w/v) formalin solution (column a) or in a 4% (w/v) formalin solution in saline (column b and c). Before incubation, they were washed three times in 5-10 ml of saline and twice in a standard Krebs-Ringer medium. They were then drained on a glass surface (McIlwain & Rodnight, 1962 p. 116), reweighed (column a and c), preincubated and incubated for 40-60 min. After incubation, the slices were again drained as described and reweighed. The swelling was calculated as a percentage (w/w) of the fresh weight. The values presented are the means $\pm$ S.D. of the numbers indicated.

Statistics:

*** P < 0.01 **** P < 0.001. The figures underlined are not significantly different. The statistical comparisons were complicated by unequal variances of the swellings between a) and b) c). Therefore, a modified t-test (Bailey, 1959) was used in these cases. A paired t-test was used in the comparison between values in rows x) and y) because the weights of each slice were recorded during the procedures. The underlinings are based on the analysis of variance. The slices b) and c) are the same as the fixed slices in Table 2.11 in which none of the groups were significantly different.

TABLE 2.10 THE EFFECT OF VARIATIONS OF THE MEDIUM ON Li^+ IN INCUBATED CEREBRAL SLICES,

UNFIXED AND FORMALIN FIXED

variable	medium	fixed slices	unfixed slices	total no. of samples	overall significance (P values)	overall S.E.M.
a) glucose (mM)	10 or 0	10 0	0 0	10		
Li^+ (mmol/l or kg)	1.392	<u>1.504</u> <u>1.574</u>	1.657 <u>1.833</u>	24	< 0.001	0.0295
b) Ca^{2+} (mM)	2.6 or 0	2.6 0	2.6 0			
Li^+ (mmol/l or kg)	1.519	<u>1.704</u> <u>1.816</u>	1.873 <u>2.749</u>	24	< 0.001	0.0627
c) Ca^{2+} (mM)	0	0 0	0 0	0		
and glucose (mM)	10 or 0	10 0	0 0	10		
Li^+ (mmol/l or kg)	<u>1.544</u> *	<u>1.937</u> <u>1.969</u>	<u>1.689</u> * <u>3.215</u>	24	< 0.001	0.0471

Cerebral slices which had previously been fixed in formal-saline and rinsed were incubated for 40 min with slices prepared in the usual way, one pair of slices in each incubating beaker. Except for the media (8 samples), each of the Li^+ concentrations presented is the mean of 4 samples and is referred to incubated weight. The analysis of variance and a multiple range test for 1% significance was used (Duncan, 1955). With one exception*, any two means not underscored by the same line are significantly different, any two means underscored by the same line are not significantly different.

TABLE 2.11 THE EFFECT OF VARIATIONS OF THE MEDIUM OF K^+ AND Na^+ IN INCUBATED CEREBRAL SLICES,
UNFIXED AND FORMALIN FIXED

variable	unfixed slices			fixed slices			total no. of slices	overall significance (P values)	overall S.E.M.
a) oxygen	O_2	N_2		N_2	O_2				
K^+ (mmol/kg)	<u>56.2</u>	<u>15.4</u>		<u>10.1</u>	<u>7.9</u>		16	< 0.001	1.17
Na^+ (mmol/kg)	<u>104.6</u>	<u>150.7</u>		<u>158.4</u>	<u>166.2</u>		16	< 0.001	2.46
b) glucose (mM)	10	0		10	0				
K^+ (mmol/kg)	<u>74.5</u>	<u>27.8</u>		<u>7.5</u>	<u>8.5</u>		16	< 0.001	4.32
Na^+ (mmol/kg)	<u>112.5</u>	<u>156.5</u>		<u>176.3</u>	<u>182.8</u>		16	< 0.001	4.51
c) Ca^{2+} (mM) and	0	0		0	0				
glucose (mM)	10	0		10	0				
K^+ (mmol/kg)	<u>48.3</u>	<u>14.5</u>		<u>9.8</u>	<u>9.5</u>		16	< 0.001	0.964
Na^+ (mmol/kg)	<u>122.0</u>	<u>151.5</u>		<u>188.8</u>	<u>189.8</u>		16	< 0.001	6.07

Each of the Na^+ or K^+ concentrations presented is the mean of 4 samples. A multiple range test for 1% significance was used. Further experimental and statistical details are given in the legend of Table 2.10.

TABLE 2.12 THE EFFECT OF VARIATIONS OF THE MEDIUM ON THE SWELLING OF INCUBATED CEREBRAL SLICES

UNFIXED AND FORMALIN FIXED								
variable		unfixed slices		fixed slices		total no. of slices	overall significance (P values)	overall S.E.M.
a)	oxygen swelling (%)	O ₂ <u>12.7</u>	N ₂ <u>32.4</u>	O ₂ <u>29.8</u>	N ₂ <u>29.0</u>	16	< 0.001	1.71
b)	glucose (mM)	10	0	0	10			
	swelling (%)	<u>15.8</u>	<u>33.8</u>	<u>27.5</u>	<u>24.4</u>	15	< 0.001	1.87
c)	Ca ²⁺ (mM)	2.7	0	2.7	0			
	swelling (%)	<u>18.2</u>	<u>25.2</u>	<u>23.7</u>	<u>29.1</u>	14	n.s.	1.38
d)	Ca ²⁺ (mM)	0	0	0	0			
	glucose (mM)	10	0	10	0			
		<u>15.0</u>	<u>63.3</u>	<u>25.0</u>	<u>27.0</u>	15	< 0.001	1.62

Cerebral slices were weighed before, and after incubation, as described elsewhere in this section. The values presented express the increase in weight after incubation as a percentage of the fresh weight, and are the means of 3-4 slices. A multiple range test for 1% significance was used. Further experimental and statistical details are given in the legend of Table 2.9 and 2.10.

2) THE MEASUREMENT OF Li^+ , K^+ AND Na^+

In the previous section several measurements of Li^+ , K^+ and Na^+ were presented. In most cases it was assumed that these values were true, and we examined whether isolation and incubation might obscure the primary effects under investigation. The main intention of these experiments was to demonstrate changes in concentrations rather than absolute values of the cations. The aim of the present section is to find out whether the signals shown by the measuring instruments are representative of the true concentrations in the samples, i.e. to determine the accuracy of the measurements.

The method used in the measurement of K^+ and Na^+ in the present thesis was flame photometry, based on atomic emission. Li^+ was measured by atomic absorption spectroscopy. Both methods are well established and there are several textbooks in the field. The following description owes much to Margoshes' 'Introduction to Flame Photometry' (1962) whose classification of sources of error has been used. The textbook, 'Atomic Absorption Spectroscopy' by Christian & Feldman (1970) was often consulted when practical and theoretical problems arose.

Atomic emission is based on the fact that elements heated in a flame will emit light at characteristic wavelengths. The resonance line, corresponding to the lowest excited state, usually contains the strongest light since this transition is the most frequent. Atomic absorption makes use of the concomitant phenomenon of absorption of light at the particular wavelength by atoms in the ground state. Atomic emission and absorption are complementary phenomena and the principle in one method may be a source of error in the other. In atomic absorption, the function of the flame is to vaporize the sample and to bring into the atomic form a reproducible proportion of the element to be measured. The light signal that is sent

through the flame contains only the wavelengths of the element to be measured. The absorption of this signal depends on the atomic concentration of the element in the flame. The detection system is made insensitive, by various devices, to the emitted light from the sample. As a consequence, emission from other elements (radiation interference) is avoided in measurement by atomic absorption. The other advantage of atomic absorption is the ability of the method to measure elements that do not emit light at the usual flame temperatures.

The principles of atomic emission and absorption are easily understood, but the reliable quantitation of an element in a biological specimen by these methods is less straightforward. The use of expensive equipment may greatly reduce the known sources of error in the methods. However, usually the question is not how to get the best instruments available, but how to get the best from the instruments available. These were a flame photometer (Evans Electroselenium Ltd., EEL) for the K^+ and Na^+ measurements and an atomic absorption spectrophotometer (Perkin-Elmer 303) for the Li^+ measurements. The fuel was propane and acetylene, respectively. With the exception of radiation interference and self-absorption, which are specific errors of atomic emission, the sources of errors are common to both methods and, therefore, are most conveniently treated together by looking at the steps of the measurement. Errors could arise i) in the preparation of standards or samples, ii) in the atomizer, iii) in the flame, iv) in the monochromator and detector. In practice, there will often be overlap. The question of the linearity of the standard curves, is best treated separately v).

i) The preparation of standards or samples

In the end, most chemical measurements, irrespective of methods, are based on standards made up of pure chemicals that were accurately estimated by weight. In the present experiments all chemicals used were analytical reagents

(British Drug House Ltd.). The carbonates of Li^+ and Ca^{2+} were dissolved in HCl to make 1 M stock solutions as the chlorides are strongly hygroscopic (Amdisen, 1967). A slight excess in acidity had no effect on the pH in the final standard solutions which were modified by the addition of TCA or a Krebs-Ringer solution, as mentioned below. The chlorides were used as standards for the Na^+ and K^+ measurements. The balance (Sartorius, type 2642) was serviced regularly. Volumetric equipment was controlled by weight in critical measurements, and micropipettes were used for small amounts (Oxford International Sampler Micropipettes System).

The great sensitivity of even the simplest flame photometer to Na^+ is a mixed blessing. Generally, it is not necessary to use it at maximum sensitivity, and it means that contamination with this ubiquitous ion presents a serious problem. However, a less sensitive line than the resonance line could not be used with the equipment available. Also, there was not enough material to allow flushing with the sample of the test tubes used for the final dilutions. Therefore, the sensitivity was decreased by diluting the samples less and using more concentrated standards even though this meant that the standard curves were no longer linear (Table 2.18). Nevertheless, contamination of glassware was found to cause unpredictable errors in the initial experiments in spite of the lower sensitivity and standard washing procedures. Thus as a control during routine measurements, distilled water was examined for Na^+ in 87 tubes, and 22 (25%) gave readings that were equal to or higher than 10% of the signal from the lowest standard of 200 μM . Contamination of glassware with K^+ and Li^+ was controlled in a similar way, but was found to be without any significance. Any similar contamination of glassware, by any of the ions in question, at an earlier stage in the preparation would be of no consequence for the measurements because of the subsequent dilution by 10-200

times. Therefore, the test tubes used for the final dilution were filled with distilled water, checked for the absence of Na^+ and dried prior to use, whereas all other glassware went unchecked through the same washing procedures.

Trichloroacetic acid 6% w/v (TCA 6%) was used for the extraction of electrolytes from the cerebral tissue (McIlwain & Rodnight, 1962, p. 20). It was found to depress the Li^+ signal whereas cerebral tissue had the opposite effect (Table 2.13). Therefore, both were added to the Li^+ standards. However, TCA has been reported to cause retention of Li^+ in precipitates (Coombs, 1971), i.e. a true decrease in the Li^+ concentration which would not be corrected for in the above preparation of the standards. Also, storage of the samples, which was necessary for practical reasons, is known to affect the concentration of diluted samples. These questions were examined together (Table 2.14). There was no effect of homogenization or precipitation. The average difference in recovery of Li^+ on the first day and on the fifth day of storage was 1%; though statistically significant, it was considered trivial and disregarded. In both experiments (Table 2.13 and 2.14) there was a significant difference between the mean recovery of the lowest standard and that of all the others. This is not surprising, because any absolute changes in the signal causes the greatest relative change in standards of low concentration. It indicates the necessity of making up standards for small concentrations even if the standard curves are linear (Table 2.18).

TCA caused an increase in the K^+ and Na^+ readings (not shown). Consequently, TCA was added to the Na^+ and K^+ standards. The effect of cerebral tissue on the readings had to be assessed indirectly and will be discussed later.

ii) Errors arising in the atomizer

The magnitude of the signal is influenced by the number and size of droplets of the sample in the flame. These are again

dependent upon the viscosity, density and surface tension of the sample, which may differ considerably from distilled water with respect to these characteristics. This is one of the reasons for making the standards as similar to the samples as possible ('matrix matching'). It was noted that the solutions of TCA were aspirated more slowly by the suction systems than solutions of distilled water. This might explain the depression of the signals of Li^+ in TCA, but not the effect of TCA on the Na^+ and K^+ signals. The rate of suction of the sample into the apparatus is also influenced by the distance of the opening of the suction tube from the surface of the sample. Therefore, this distance was always kept as constant as possible.

iii) Errors arising in the flame

The measurement of Li^+ by atomic absorption is free of interference from emission by other elements. Nevertheless cation interference with the Li^+ measurements is known to take place when atomic absorption is used (Amdisen 1975). This is because Li^+ is easily ionized in the flame and the absorption of light by the ion is less than that by the atom. The presence in the flame of another easily ionizable element like K^+ or Na^+ reduces the degree of ionization of Li^+ by mass action of the electrons. In the presence of a Krebs-Ringer solution, diluted like a sample to be measured for Li^+ , there was an increase in the signal of more than 5% (not shown). Therefore, a Krebs-Ringer solution was added to the standards in the same proportion as that of the samples. It was later found that a solution of 0.9% saline, suitably diluted, was an adequate substitute for the Krebs-Ringer solution in the measurements. The amount of Li^+ in the slices was small compared to the amount of tissue and the other cations. Furthermore, the content of K^+ and Na^+ of the slices varied considerably in the experiments of Chapter Four and Five. Therefore, studies of the recovery of Li^+ were carried out under all experimental conditions using the standards already

mentioned. The recovery was shown to be independent of the media with respect to K^+ , Na^+ , Ca^{2+} and Mg^{2+} (Table 2.15).

In the measurement of Na^+ and K^+ , radiation interference from one of the elements with the other should be minimal because the resonance lines are so far apart, 589 and 766/770 nm, respectively. The standards used for measurements of Na^+ and K^+ in cerebral slices had a concentration ratio of 2:1 with respect to Na^+/K^+ because this was their ratio in cerebral slices under most conditions (Cummins & McIlwain, 1961). However, the Na^+ and K^+ in the slices varied considerably in some of the experiments, as mentioned above. Therefore, the influence of K^+ on the Na^+ signal and the influence of Na^+ on the K^+ signal was examined. It was found that K^+ caused a slight depression of the Na^+ signal at the ratio of concentrations normally found in cerebral slices so that metabolic changes causing low concentrations of K^+ would give Na^+ readings that were too high by a maximum of 4% (Table 2.16). There were no systematic changes of the K^+ signal within realistic concentration ratios of Na^+/K^+ (Table 2.17) and the Na^+/K^+ ratio of 2:1 in the standards was used in all measurements.

Interference may be due to factors other than elements in the flame. Nonspecific reduction of the brightness of the flame can be caused by light scatter from solid particles and unevaporated solvent drops or unburnt carbon particles. In atomic absorption this would give a falsely high signal because of increased absorption, in atomic emission it should cause a lowering of the signal. The recovery experiments (Table 2.14 and 2.15) showed that a satisfactory matrix matching was obtained by the standards used for the Li^+ measurements.

Matrix effects could not be assessed directly in the measurements of K^+ and Na^+ . In an attempt to distinguish matrix effects from radiation interference, rat cerebral tissue was homogenized in distilled water. The homogenate was then

dialysed at 4°C against 500 volumes of distilled water with 4 changes over 24 h. The protein-rich supernatant was added to aqueous K^+ and Na^+ standards in a final dilution corresponding approx. to that of the cerebral tissue in the usual samples. There was no interference with the K^+ readings, whereas the Na^+ readings showed an increase of 1-2% of the signal. As mentioned, one would have expected, if anything, a decrease in the signal of both the cations. There was no independent way of checking the dialysis and so it may well have been due to Na^+ left in the dialysate. However, the effect was small enough to be disregarded.

iv) Errors in the monochromator and detector

The use of a good monochromator (Perkin Elmer 303) ensures that little light outside the frequency of the resonance line is transmitted to the photocell. However, the resonance lines of Na^+ , Li^+ and K^+ (589 nm, 671 nm and 766/770 nm, respectively) are so far apart that the broader lines from filters (EEL) are sufficient for the K^+ and Na^+ measurements. Also, apart from the relatively small concentrations of Ca^{2+} present in the slices no other elements should cause radiation interference with the K^+ and Na^+ measurements, as can be seen from a table of flame emission spectra (Documenta Geigy, 1962, p. 270).

Drift in the electronic circuitry could be controlled by frequent checks with the standards. The dark current noise determined the sensitivity of the Li^+ measurements. Thus for Li^+ , a signal to noise ratio of 2 (detection limit) was found when the concentration in the final solution was 2 $\mu\text{mol/l}$. In the kinetic experiments (Chapter Three, Six, and Seven) amounts below 5 $\mu\text{mol/l}$ in the final solution had to be measured sometimes. There were the same problems of drift in the measurements of K^+ and Na^+ , whereas even the lowest concentrations in the samples were far from the detection limits.

v) Linearity of the standard curves

Blijenberg & Leijnse (1968) compared measurements of Li^+ in serum by atomic emission and atomic absorption spectroscopy. Using atomic absorption and final concentrations similar to those of the present experiments, the authors found a calibration curve that was clearly non-linear. In the present experiments, standard curves were used routinely. It was estimated whether non-linearity was present and, if so, whether it would be of any practical relevance for the measurements. This was done by calculating the deviations of the standards from a fitted straight line through all of them. Similar calculations were made for the K^+ and Na^+ standard curve (Table 2.18). The joint standard curve for Li^+ showed a random distribution of the mean values of the standards around the fitted line. The Na^+ curve was clearly non-linear. The K^+ curve showed a less pronounced deviation of the low end.

Thus the use of standard curves might seem unnecessary in the measurement of Li^+ , and perhaps K^+ . However, as mentioned earlier, the low Li^+ standards showed some differences in recovery, and statistically these standards showed signs of inhomogeneity. It was also found that calibration curves with high and low values sometimes showed systematic deviations from the 'average' curve. Finally, the measurements were usually carried out once a week and often included more than a hundred samples of widely differing concentrations. Therefore, the inclusion of a few more standard samples took very little additional time. If there were signs of deviations at extreme concentrations, a fully adequate linear fit could then be obtained by the use of restricted parts of the curves for these high or low concentrations.

In conclusion, it can be stated that a satisfactory matrix matching was obtained in the Li^+ measurements to allow for a large range of Na^+ and K^+ concentrations in the samples, and

that retention due to coprecipitation was not observed in the use of TCA. Simultaneous measurements of evaporation and Li^+ in the medium reflects the accuracy of the measurements (Table 2.3b). This was also true in the case of Na^+ and K^+ (Table 2.3a). The accuracy of these measurements in the tissue was more difficult to check, but the interference of K^+ on the Na^+ measurements was small and there was none of Na^+ on the K^+ measurements, tissue constituents that were water soluble and non-dialysable had a negligible influence on the measurements and the effect of TCA was made up for in the standards.

TABLE 2.13

THE INFLUENCE OF TCA AND CEREBRAL TISSUE
ON THE Li⁺ SIGNAL

final Li ⁺ concn ($\mu\text{mol/l}$)	TCA recovery (%)	TCA + brain recovery (%)
10	91	100
20	84	93
30	90	93
40	84	90
50	87	92
60	87	92
70	87	93
80	84	89
90	88	90
100	89	94

Each figure represents one Li⁺ standard prepared in either 6% TCA or 6% TCA containing fresh rat brain, diluted approx 1 : 15. The readings of the standards were compared with the readings of corresponding standards made up in distilled water (100%).

The analysis of variance showed an effect of cerebral tissue on the Li⁺ signal ($P < 0.001$) and an effect of concentration ($P < 0.01$), which was caused by the standards, 10 $\mu\text{mol/l}$ in Li⁺.

TABLE 2.14 THE RECOVERY OF Li^+ IN THE PRESENCE OF INCUBATED
CEREBRAL SLICES HOMOGENISED IN TCA, AND THE
EFFECT OF STORAGE OF THE SAMPLES

final Li^+ concn ($\mu\text{mol/l}$)	series A recovery (%)		series B recovery (%)	
	1. day	5. day	1. day	5. day
10	100	103.5	107	105
20	101	103	98	103
30	99	101	100	100
40	101	102	96	99
50	99	100	100	101
60	99	100	100	100
70	99	101	99.5	100
80	100	100	98	99
90	-	100	-	101
100	101	101	100	100

Cerebral slices were incubated for 1 h in a standard medium without Li^+ and drained. The final Li^+ concentrations were obtained by adding LiCl solutions to the tissue before (series A) or after (series B) homogenisation and precipitation with 6% TCA. Each figure represents one sample read against a routinely prepared standard containing fresh rat brain, on the day of the experiment, and after storage at 4° for 5 days.

The analysis of variance showed an effect of the Li^+ concentration ($P < 0.01$), which was caused by the standards, $10 \mu\text{mol/l}$ in Li^+ , and an effect of storage ($P < 0.05$), but no effect of homogenisation.

TABLE 2.15 THE EFFECTS OF OTHER CATIONS ON THE RECOVERY OF Li^+ IN THE PRESENCE OF INCUBATED CEREBRAL SLICES

K^+ (mM)	1.0	2.5	3.7	7.5	10.0
recovery (%)	101.2 ± 1.8	101.7 ± 1.2	99.2 ± 1.7	101.3 ± 1.2	101.2 ± 0.8
Na^+ (mM)	120	130	140	160	170
recovery (%)	101.2 ± 1.1	99.8 ± 0.7	99.8 ± 1.0	100.0 ± 1.1	100.4 ± 0.5
Ca^{2+} (mM)	0	1.3	2.6	3.9	5.2
recovery	98.8 ± 0.7	99.8 ± 1.2	99.5 ± 0.7	99.8 ± 1.2	100.0 ± 1.3
Mg^{2+} (mM)	0	0.95	1.30	1.95	2.60
recovery (%)	100.9 ± 0.8	99.1 ± 1.1	100.0 ± 0.9	99.7 ± 1.5	99.3 ± 1.4

Cerebral slices were incubated without Li^+ in the presence of the concentrations of cations shown in the table. The final Li^+ concentration in all samples was $60 \mu\text{M}$. In all other respects, the samples were treated as stated in the legend of Table 2.14. There was no significant effect of homogenisation. Therefore, results from series A and series B were pooled. Values presented are the means $\pm$ S.D. of four slices. The analysis of variance showed no effect of cations.

TABLE 2.16
THE INFLUENCE OF K^+ ON THE Na^+ SIGNAL

final concn of K^+ (mmol/l)	0	0.05	0.1	0.2	0.4	0.8	1.6	3.2
K^+/Na^+ ratio	0	1/10	1/5	1/2.5	1/1.25	1.6/1	3.2/1	6.4/1
recovery (%)	(100)	100	100	96	96	96	96	93

TABLE 2.17
THE INFLUENCE OF Na^+ ON THE K^+ SIGNAL

final concn of Na^+ (mmol/l)	0	1.75	2.5	5.0	10.0	20.0	40.0	80.0
Na^+/K^+ ratio	0	3.5/1	5/1	10/1	20/1	40/1	80/1	160/1
recovery (%)	(100)	99	100	98	100	98	95	91

Solutions containing fixed concentrations, 0.5 mmol/l, of either NaCl (Table 2.16) or KCl (Table 2.17) in distilled water, and increasing concentrations of either KCl or NaCl, were measured in an EEL flame photometer. Each figure represents a solution, made up as described, which was read twice in a randomized sequence against a solution containing only NaCl or KCl, 0.5 mmol/l (100%).

TABLE 2.18

THE LINEARITY OF STANDARD CURVES

final concn
of stds.
($\mu\text{mol/l}$)

Comparison of reading with 'true' value
(%)

Li^+	K^+	Na^+	Li^+	K^+	Na^+
10			101.7 ± 2.8		
20	100	200	100.2 ± 2.8	96.6 ± 2.3	90.7 ± 3.9
30			99.4 ± 2.0		
40	150	300	99.2 ± 1.7	100.9 ± 1.7	100.5 ± 2.8
50	175	350	100.0 ± 1.5	100.7 ± 3.4	102.1 ± 4.3
60	200	400	99.8 ± 1.4	100.0 ± 3.0	103.2 ± 2.8
70	225	450	100.0 ± 0.9	102.0 ± 2.1	103.7 ± 3.4
80	250	500	101.4 ± 0.9	100.2 ± 1.4	99.7 ± 1.6
90	275	550	99.8 ± 0.8	99.1 ± 3.3	99.1 ± 2.6
100			99.8 ± 0.8		
110	325	650	99.7 ± 1.1	99.5 ± 2.9	98.1 ± 1.8
120			100.0 ± 0.8		

Straight lines were calculated by means of the readings of the standards for the Li^+ , K^+ , and Na^+ measurements. The individual readings on the ordinate were then compared with their 'true' value (final concn.) on the abscissa, using the projections from the straight line. A perfect fit would be 100% for all values. Each value presented is the mean $\pm$ S.D. of ten to twelve readings taken from routine experiments.

3) CALCULATION OF THE INTRACELLULAR SPACE

The precautions mentioned in the last section serve to make sure that the amounts of a substance measured in a prepared sample are as near as possible to the true value in that sample. If the sample originates from a suspension of cells we may furthermore be reasonably sure that we are measuring the intracellular contents of that substance, in a known dilution. However, in the preparation of a composite tissue for measurement the substance is released from inside the cells and diluted with an unknown amount of medium-like fluid from the extracellular space or adhering to the surface of the tissue. Instead of an intracellular amount we measure an average value of concentrations that may be very different. Therefore, it would be desirable to relate concentrations to the volume of fluid inside the cells.

In order to calculate the intracellular space we must know i) the amount of solids , ii) the extracellular space, and iii) the amount of adhering fluid.

i) The amount of solids

This amount is usually stated as a dry weight, which is obtained by removal of the water from the tissue in an oven at approx. 100°C, or by freeze-drying (McIlwain & Rodnight, 1962 p. 17; Lund-Andersen & Hertz, 1970). Heating may increase the dry wt. because of oxydation, but may also decrease it because of loss of volatile substances other than water. However, these sources of error cannot be important because tissue water measured by incubation with tritiated water is essentially the same as the water content measured by drying slices (Cohen et al., 1968). Therefore, the weight of the water in the cerebral slice can be calculated by subtraction of the dry weight from the total weight. The dry weight of fresh rat cerebral cortex has been measured by several authors and found to be 18-19% (Hertz, 1973, p.

63); this value is reduced by the swelling of the slices. It is usual to assume a specific gravity of 1 for the tissue water and to operate with spaces in terms of volumes. This is not so for the solids in the tissue, but it is possible to get a reasonable estimate of their volume. Tabulated values for solutions of salts and proteins exhibit linearity between the amount of solute and the specific gravity of the solution. Considering the solutions ideal at higher concentrations, the specific gravity of a fictive '100% solution' can be calculated and hence the reciprocal value, the specific volume. For sodium chloride, 1-26% w/v, the specific volume that can be inferred in this way is 0.57 (Documenta Geigy, 1962, p. 319). The proteins of serum show a specific volume of 0.76 (Sunderman, 1936, equation 2). Most of the solids of a cerebral slice are not in a free solution, but are parts of cellular structures and probably take up less space than indicated by the figures above. However, the volume of solids is immaterial in the experimental determination of the water content, and for all practical purposes the amount of water can be referred to interchangeably in corresponding units of weight or volume. The question of adsorption of substances to the solids of the cerebral slice was dealt with in section 1Biv) (Table 2.8).

ii) The extracellular space

In a comprehensive review, 'the estimation of extracellular space of brain tissue in vitro' (Cohen, 1972), various possible methods are described and assessed, a) electron microscopy, b) measurement of the electrical resistance of the tissue, c) efflux kinetics of markers, d) the use of extracellular markers.

a) Electron microscopy: The principal objections to this method are the dependence of the estimated space on the preparation of the tissue and the lack of accepted criteria

for the choice of preparation. 'By a proper choice of methodology one can produce any desired value by electron microscopy' (l.c., p. 216). Practical difficulties would prevent the method from being used routinely in experiments.

b) Measurement of the electrical resistance of the tissue: In this method the underlying assumption is that the electrical current used in resistance measurements on tissues is mainly carried by the extracellular fluid. Therefore, decrease of resistance (increase of conductance) is interpreted as an increase in the extracellular space. The theoretical objections to the method are that an increase in cell volume also causes an increase in the average path length of the current and creates blind pockets of non-conducting extracellular fluid, that the interpretation depends on the cell geometry, which is complex in nervous tissue, and that blood vessels act as shunts for the current. As a practical objection of some weight the author refers (his Table V) to the considerable differences in the calculated extracellular spaces arrived at by different authors under apparently similar conditions.

c) Steady state efflux kinetics of markers: This method obviates the cardinal requirement of method d), namely that the marker must be excluded from the cells. Detailed descriptions of the compartmental analysis of the washout curves are available (e.g Solomon, 1960). It is essential that the intracellular efflux is slow compared to the diffusion from the extracellular space and that the tissue does not change during the total period of saturation and efflux. The latter condition may be difficult to fulfil. The author mentions as further disadvantages, the number of measurements necessary, and the experimental scatter which makes extrapolations uncertain. One may argue that this kind of uncertainty can at least be indicated in precise terms, but clearly the method is unsuited for routine

measurements.

d) The use of extracellular markers: The advantage of some of these markers is the ease of their practical use. They can be added to the incubation medium under all experimental conditions, and can be measured in cerebral slices together with the substance under investigation, because radioactive labelling minimizes their concentration and the necessary amount of sample. Provided that the measurements of the marker are technically satisfactory, the calculation of an apparent volume of distribution, with the medium as a reference fluid, requires no assumptions. However, if such an apparent volume of distribution is interpreted in an anatomical sense, then some basic features of the markers are fundamental for their use.

In the review (Cohen, 1972, p. 183), the author states twelve requirements for an ideal marker. It is tempting to apply statistical reasoning to such a statement; 'the application of multiple conditions can greatly decrease the chances of success; failure to realize this soon enough has lead to a great many people remaining single!' (Parker, 1973, p. 4); or going on looking for the ideal marker. On the other hand, it would be perfectly valid to use several markers that satisfied only some, but different, requirements. Identical results would strongly support the concept of an anatomical space. However, the second and third requirement in the review, namely free access to all extracellular liquids and complete exclusion from the cells, must be essential to all extracellular markers.

A priori, inulin could be expected to have some of the properties of the fictive 'ideal marker'. It is non-toxic, it is not native to the tissue, it is not metabolized and does not affect metabolism, and it is easy to determine; first and foremost, it has a large molecular size. However, the molecular size of a polysaccharide is an average value. Incomplete access to all parts of the extracellular space of

some molecules and access to the cells of others might invalidate the measurements if the heterogeneity were prominent. Determination of fructose and fructosans in samples of inulin by paper chromatography showed that about 25% had molecular weights of less than 1000 and that 10 - 20% was fructose. Even after recrystallization there was a pronounced polydispersity, as demonstrated by gel filtration (Phelps, 1965). In renal clearance studies, the heterogeneity of particle size was related to the pattern of excretion, showing the problem to be of practical significance (Bassir, 1956).

While the problem of inhomogeneity of inulin may be solved by careful techniques (Cohen, 1972, p. 196), it is difficult to dismiss the following evidence of intracellular penetration of inulin (1 - 7).

(1) In efflux experiments, 5 - 10% of the inulin remained in leech nerve cord after prolonged washing. Autoradiography demonstrated location in the neurons (Nicholls & Wolfe, 1967). (2) After intraarterial injection of ^3H -inulin into cat sympathetic ganglia, 10 - 15% of the activity was found in the neurons by autoradiography. False traces due to background, chemical - or pressure artifacts, spread of radiation, and translocation of inulin during the preparation, were carefully excluded by control experiments (Brown et al., 1969). (3) Varon & McIlwain (1961) found that inulin diffused out of cerebral slices slowly and incompletely. (4) In experiments by Pappius et al. (1962), approx. 12% of the inulin was left in the slices after 120 min in inulin-free media. (5) Cohen et al. (1968), in cerebral slices, demonstrated the presence of a space that was accessible to inulin at 37°C , but not at 0°C . Inulin that had entered this 'second inulin space' became trapped when slices were transferred to a medium of 0°C without inulin. None of these phenomena were due to differences in the rate of diffusion. (6) Schousboe & Hertz (1971) showed that 10%

of the efflux of inulin from cerebral slices came from a slowly exchanging compartment. (7) By means of carefully controlled steady state efflux kinetics of inulin from cerebral slices, the presence of a compartment was demonstrated, which had a half-time of about a hundred times greater than that of free diffusion. The relative size of the compartment was independent of the thickness of the slices. It was at least 15% of the total inulin space, the uncertainty being due to the lack of equilibrium even after 60 min. The homogeneity of the inulin was checked (Lund-Andersen, 1974).

Not all the authors mentioned above, did themselves interpret their experiments as evidence of penetration of inulin into cells. However, jointly, these experiments strongly challenge the two crucial assumptions in the use of inulin as an extracellular marker. Even assuming that the 'intracellular' compartments demonstrated by kinetic methods have no anatomical correlates, a similar kinetic behaviour caused by adsorption to extracellular surfaces would make inulin equally ill suited as indicator of an anatomical space. Sucrose, which is sometimes used as an alternative to inulin, indicates a larger 'extracellular' space than does inulin under a variety of experimental conditions (Pappius & Elliott, 1956a; Hertz et al., 1970); therefore, by implication, it cannot be considered exclusively extracellular. A priori, this must be assumed for all carbohydrates with a molecular weight lower than that of inulin.

In the review (Cohen, 1972), the author advocates the use of extracellular markers. However, his reservations about inferences arising from their use are tantamount to an outright rejection of their use: 'the decision to include, exclude, or ignore the existence of the second space, like the choice of a marker, defines a conventional extracellular space which bears an unknown relation to the so far unmeasurable "true" extracellular space'. (l.c. p. 193 f.).

The use of inulin and other markers in the more advanced technique of steady state efflux kinetics is quite legitimate, as was explained earlier. Also, during brief contact between slices and markers, the slow intracellular penetration of inulin into intact cells is of no consequence. This use of inulin was mentioned in connection with the cutting procedure(1Aii))and will be described in the next section on the adhering fluid.

iii) The amount of adhering fluid

In the use of standard draining techniques (McIlwain & Rodnight, p. 116), a film of adhering medium inevitably remains on the slice. The effect on the final average concentration of a substance located intracellularly is similar to that of the extracellular fluid, and depends on the concentration ratio of the substance between the cells and the medium. Also, from the end of the incubation until the beginning of the preparation for measurement, there is an unknown degree of concentration of the medium due to evaporation of water when the slices are handled in the laboratory atmosphere. Whether these effects are significant or trivial depends on the volume of adhering medium. Consequently, several attempts have been made to assess its magnitude and nature. In the three papers, mentioned below, the approaches were different.

Varon & McIlwain (1961) found an average weight increase of approx. 10% in cerebral slices after brief contact with media at room temperature, followed by a standard draining procedure. Slices cut on two sides showed a larger amount of water, as estimated by weight increase and inulin, than slices with one intact surface when they were incubated for 60 min. The obvious interpretation of this difference would be uptake of water into parts of the slices that were damaged in the cutting procedure.

Pappius et al. (1962) assessed the cell damage microscopically. The authors found that the apparent spaces of inulin and fluorescence-labelled protein were similar in size after 60 min incubation. From the visualized distribution of the labelled protein on the surface of the slices and the similarity in size of the two spaces, the authors inferred that the inulin space was representative of damaged regions of the slices. The adherent fluid assessed in this way, would amount to approx. 50% of the total fluid in the slice. However, the protein space kept expanding from 15 min until it was no longer measured after 180 min, whereas the inulin space in the authors' experiments did not (Pappius & Elliott 1956a). The possibility of redistribution of the protein during preparation for microscopy was not considered.

Cohen (1974a), as the basis of his evaluation of the adherent fluid considered a geometric model in which several simplifying assumptions had to be made. The model would predict a linear relationship between the reciprocal slice thickness and adherent fluid, because the exposed surface area increased with the number of slices per unit of tissue. From the linear regression coefficient, and the extrapolation to zero thinness corresponding to an intact slice of infinite thickness, the thickness of the fluid film on each side of the slice could be calculated, assuming isotropic swelling. In the experiments, slices of 0.16 mm to 0.69 mm were exposed to media for a few seconds ('initial minimal contact') either without markers or in the presence of inulin or sucrose. Brief exposure to the markers also took place after incubation for 60 min ('delayed minimal contact'). The media were 0°C or 37°C. The film thickness at 0°C, as estimated by the increase in weight, was 0.0056 mm, but was higher at 37°C. This was contrary to expectations, because surface tension and viscosity would decrease at 37°C and thus make the film thinner. The difference was

interpreted as being due to superficial swelling, and the value at 0°C was taken as a maximum value for the 'true' film thickness. The film thickness which could be estimated by delayed minimal contact with inulin and sucrose at 37°C was 0.022 mm and 0.035 mm, respectively. In the case of inulin, identical results were obtained if the film thickness was measured by means of the loss of marker produced by rinsing the slices for a few s. The difference between film thickness estimated by markers and by weight increase was interpreted as being due to an outer layer that could be rapidly displaced by fresh incubation medium and an inner, unstirred layer of partially damaged cells that could equilibrate with the medium only by diffusion. This layer would only be estimated by weight if accompanied by swelling.

The increase in weight due to adhering medium could only be measured initially, because it could not be distinguished from swelling after 60 min. However, the points in the estimation of the corresponding marker spaces after initial minimal contact are not randomly distributed about the fitted straight lines (l.c. Fig 2 & 3, standard media). Therefore, under these circumstances the model cannot be valid, and subtraction of water space from the marker spaces (l.c. Table 1, last column) can only be considered a qualitative expression of the superficial damage produced by slicing. The geometrical model, by necessity, assumes homogeneity of the tissue. However, it would seem likely that the initial changes on the surface of the slice take place more rapidly in a thin slice than in a thick one, whereas these differences even out after a period of incubation. This would explain the better fit to the straight lines of the values obtained after delayed minimal contact.

As can be seen from the papers referred to above, the concept of adhering fluid is not a simple one. The interpretation of quantitative data of even an elaborate approach

leaves much to subjective judgement. In the estimation of the volume of intracellular fluid by 'extracellular' markers, ii), matters are complicated further by the inclusion of a layer that is neither extracellular nor intracellular and is penetrated to different depths by various markers. According to the previous calculations (Cohen, 1974a), the 'true' proportion of the adhering fluid in a 0.5 mm thick slice should be $2 \times 0.0056 \times 100/0.5 = 2\%$ of the fresh wt. (areas and specific gravities assumed equal). The 'functional' proportion would primarily depend on the number of cut surfaces, although even the intact surface may be damaged, as was mentioned earlier (Møller et al., 1974). Using the previous thickness of 0.022 - 0.035 mm, values of 5 - 7% for each (cut) surface is found. Whether one should use a value of 2%, 5 - 7% or 10 - 14% is a matter of opinion. In the present thesis, an average value of 10% of the incubated wt. has been used in calculations. One might argue that the relevant intracellular space consists of intact cells only (Pappius et al., 1962). However, the lack of a precise volume of reference would seem less serious than the consequences for kinetic experiments of having an unstirred layer of partially damaged cells outside the membrane limited intracellular space.

iv) The use of fixed slices

The experiments mentioned in ii) and iii) indicated the difficulties of finding practical and reliable methods of estimating the extracellular space and adhering fluid. On the other hand, the experiments left no doubt about the physical existence of these fluid spaces.

In Table 2.9 - 2.12 and the corresponding paragraphs the use of formalin fixed slices was mentioned. It was introduced in the kinetic experiments of Wraae et al. (1976, Fig. 1) as a structural model which was likely to have the same amount of adhering fluid and extracellular space as an unfixed cer-

ebtral slice, but no metabolism. The swelling was approx. 25% compared with 15 - 40% in the incubated slices, indicating a negligible difference in surface area. The concentration of Li^+ in fixed slices after 15 s was similar to the intercept of the kinetic curve (l.c. Fig. 1). At the time of publication of the results, this was interpreted as an indication that the intercepts of the kinetic curves were due to Li^+ in the adhering fluid and extracellular space. However, it is doubtful whether the identity of the concentrations is more than coincidental. As can be seen from the experiments (Table 2.10), which were later, chronologically, the concentrations of Li^+ in the slices after 40 - 60 min incubation were at least as high as that of the medium, probably indicating free access to the cells of Li^+ in the medium. Also, the uptake of various concentrations of Li^+ was later measured in slices fixed in pure formalin. Under these conditions, the concentration after 15 s was two times higher in relation to the concentration in the medium than was found in the slices fixed in formol-saline, mentioned earlier (not shown). The swelling of the two preparations was very different (Table 2.9), but was not related to the concentration in any simple way, and furthermore, the microscopical location of the swelling in the two preparations was not investigated. Therefore, at present, it is not found justified to use the fixed slice in the quantitative assessment of the extracellular space and adhering fluid.

v) Consequences for the results of the present thesis

It was decided to refer all concentrations of substances in cerebral slices to the final incubated wt. in the present thesis. This would be an unambiguous quantity requiring no other assumptions than those inherent in the measurement of the substances. The quantities would be available for direct comparison with the findings of other workers and at the same time could be recalculated by means of any desired correction. However, the fluid spaces do not disappear

by not being measured, and in the application and interpretation of the measured quantities their existence would have to be borne in mind.

The consequences of using an average value for the whole tissue are clear enough when substances are measured at the steady state in the uptake; a concentration ratio of the substance between the tissue and the medium of more than 0.8 - 0.9, depending on the swelling, would mean that the average value is an underestimation of the true intracellular concentration, and vice versa. In the wider application of the results the estimates can be recalculated assuming high and low volumes of the intracellular space to see whether this would alter any conclusions.

In kinetic studies, the consequences of using average values are less straightforward, because they refer to a mixture of kinetically different compartments. The existence of unstirred layers of fluid was mentioned earlier. Dainty & House (1966) examined the effects of such layers on kinetics and concluded that the problem was important for rapidly penetrating solutes. The authors used frog skin, which has a much simpler structure than the cerebral slice, but, in principle, the results should also apply to cerebral slices. Diffusion through the extracellular space may also delay the exchange of substances between the medium and the cells. The use of fully equilibrated extracellular markers assumes instant equilibrium of the solute under investigation, in addition to the other assumptions mentioned earlier (ii)). If the diffusion of the solute into the extracellular space is slow the initial concentrations in the intracellular uptake will be overcorrected by the use of the extracellular markers. The problem is similar to that of the unstirred layers. In both cases the delay due to extracellular diffusion may prevent the determination of unidirectional flux for rapidly penetrating substances and a more complicated approach is required, or extracellular dif-

fusion may be rate limiting and prevent determination of membrane limited diffusion by any method (Lund-Andersen & Kjeldsen, 1976). The opposite case, a rapidly diffusing and slowly penetrating substance, should be ideal for conventional kinetic experiments with cerebral slices. However, there is a danger in not correcting for the extracellular space if the kinetic curves are fitted by eye as a tangent to the uptake curve at zero time. Joanny et al., 1973 (their Fig. 4), clearly ignore their experimental points in fitting the uptake curves. As the initial rate of uptake was measured during the first 3 min they are likely to have overestimated this rate in passing the curve through the origin.

In the present thesis, the initial uptake was calculated by fitting regression lines to the measured concentrations. Theoretically, assuming a sufficiently slow penetration of Li^+ into the cells, this would either leave the kinetic parameters unaffected (K_m), or make them available for recalculation along the lines suggested above for the steady state (V_{max} and apparent first order rate constants) as was pointed out by Cohen (1975, p. 55, notations changed).

In practice, the choice of incubation times and the experimental scatter may present problems in the measurements, as will be discussed in Chapter Three, Five and Six, where these measurements are described.

4) THE USE OF STATISTICS

The methods of statistics make it possible for us to present results in a concentrated and instructive way. They also permit independent assessment of quantitative conclusions drawn from the results. Definitions of the concepts and description of most of the statistical methods used in the present thesis can be found in standard textbooks on statistics, some of which are referred to in this chapter. However, the technical ability to perform a statistical test,

though necessary, is not sufficient in order to draw quantitative conclusions from experimental results. Therefore, the following section will attempt to point out circumstances under which the conditions for the application of some of the well-known statistical tests are not fulfilled, and how these problems have been dealt with in the present thesis.

i) Combination of results

There are practical limits to the number of samples that can be handled in an experiment. Inclusion of 'controls', subjected to the particular conditions of the experiment except the 'treatment' under investigation, is good practice, but may not always be feasible. Therefore, we often have to combine and compare results from different experiments. Some useful working rules have been published, treating cases of increasing complexity (Cochran, 1954). In the simplest case the samples are of the same size and precision (random error) and the means are not significantly different; in the most complex case the experiments differ in all these respects. Occasionally, the differences in nominally similar experiments are so large that revision of the methods is called for rather than statistical remedy.

Most statistical tests assume homogeneity of variance. This can be tested formally on two samples by comparing the ratio of the variances with tabulated values (two-tailed F-test), and in the case of several variances by calculating the 'goodness of fit' according to a chi-squared-distribution (Bartlett, 1937). If the variations differ significantly, a suitable 'transformation', especially if the results suggest this, often makes the variances homogeneous. Other procedures based on the size and variation of the sample involve modification of the means by 'weighting' or modification of the tests by a reduction of the 'degrees of freedom' and/or extension of the 'confidence limits', mak-

ing the tests more conservative towards the 'null hypothesis'.

The homogeneity of several means is tested by the analysis of variance. If the analysis has demonstrated 'population' differences in our material the 'error variance' cannot be considered a valid expression of the variation of the combined mean in statistical comparisons. Instead, the variance between the means of the individual experiments should be used. This variance and the number of experiments is then used to calculate the standard deviation and standard error of the combined mean. This conservative estimate of the variation permits us to combine results of similar treatments in one group in spite of significant differences between the subgroups. We can then compare the group with other groups, combined or single, in the usual tests of significance. Failure to observe the above rule must be common in biological experiments. This can be inferred from publications comparing groups with a number of samples that could not possibly have been obtained in one experiment, and yet containing no other information than the outcome of Student's t-test. In a recent paper (Wraae et al., 1976; their Table 2), the corresponding paragraph at least makes it clear that the results of similar treatments have been combined, but there is no information about possible differences between the subgroups. Applying the working rules from Cochran's paper to the original data in the combined results from Table 2 it turns out that, in the Li^+ - groups, the subgroups are significantly different with respect to the Na^+ ($P < 0.05$), K^+ ($P < 0.001$) and Li^+ ($P < 0.001$); in the non Li^+ - group they are significantly different with respect to the K^+ ($P < 0.05$). The use of Student's test on the values published in Table 2 results in t values for column (b) and (c) of 3.32 and 2.47, respectively. Assuming homogeneity of the means, the authors were indeed entitled to reject the null hypothesis at the declared levels of significance.

However, using the proper estimates of variances and frequencies the t-values for column (b) and (c) become 0.82 and 0.81, both with 11 degrees of freedom, which is far from significant. As a consequence of this, the statistics in the above table have been altered in the present thesis.

Combination of subgroups of unequal size may 'bias' the joint mean by giving undue weight to small subgroups. In straightforward cases in which both the means and the variances of the subgroups are homogeneous, this can be corrected by multiplying the individual means by the ratio between the number of samples in the subgroup and the total number of samples, and adding them. However, statistical differences between the subgroups, if large, may invalidate this procedure. In Cochran's paper the matter is examined theoretically and some working rules are given, based on frequencies and the outcome of the analysis of variance.

The combined results of the present thesis were weighted without regard to the homogeneity of the means, but when checked later, according to Cochran, the differences between weighted and unweighted means were trivial. Some of the results were already published (Wraae et al., 1976). Therefore, they have been retained as published, in the present thesis. Also, in the presentation of combined results, inhomogeneity of the subgroups, in size and precision and statistical differences between them, sometimes have either not been examined or have been disregarded. In all such cases the standard deviation is based on the total sum of squares and total number of samples. This is felt justified if the intention is to present a variable that has been measured over a period, or routinely, under different experimental conditions. Such measurements usually serve the experimental worker as a check that the particular variable is reasonably well controlled, and their publication serves the reader as a quantification of 'reasonably well'. Pre-

sumably, both persons are more interested in the absolute values of the differences in such a variable than whether they are statistically significant. However, the homogeneity of the material has been examined prior to all statistical comparisons of combined results. Transformations and weighting procedures, except the simple one of the means already mentioned, will be described when they are used.

ii) Linear regression

The association between the two variables x (independent v.) and y (dependent v.) often appears to be a straight line, or it may be transformed into a linear relationship. In the analysis of linear regression the values of a and b in the equation: $y = a + bx$ are estimated from the data by the method of least squares. The advantage of predicting values of y for particular values of x is obvious, but inappropriate use of the method may make such predictions illusory.

The method assumes 1) that there is no random element in x , 2) that the variance which is not due to regression of y on x ('residual variance') is the same for all x (Campbell, 1974, p. 279 - 280). The first assumption is seldom quite true in biological experiments; e.g. the concentration of a solute in the medium may vary because of the way the solution was made up, pipetting errors and evaporation, nominally similar incubation times may vary etc., but the important consideration is that these variations be small relative to random error in y . The second assumption is often overlooked. It can be tested with Bartlett's method and transformations may equalize the variance. A typical example is that of Table 3.1 in the present thesis.

If the above conditions are fulfilled we must be able to determine the significance of the regression. Both Student's t -test and the analysis of variance can be used, or we may determine the proportion (r^2) of the variation of y that is

due to the regression on x . The square root of this proportion is the correlation coefficient. Estimated values of t , F and r require different tables for determination of their corresponding levels of significance. The r has the advantage, especially in its squared form, of giving a better idea of the scatter around a straight line than t , F or P values.

Rejection of the null hypothesis of no association between x and y does not in itself imply that their relationship is a linear one; a non-linear model may conform more accurately with the experimental data. If there are two or more different values of y at each of x , random error is represented in the variance of these replicates, and non-linearity in the scatter of their means around the straight line (Bliss 1967, p. 422 - 424). The distribution of this scatter can be calculated (Table 2.18) or inspected graphically. It may show systematic non-linear trends, or if randomly distributed, may suggest that factors independent of x are associated with change in y under the conditions of the experiment.

iii) Comparison of two means

The treatment of combined results and the tests for homogeneity of variance were mentioned in i). If the variances of two samples are significantly different, the conditions of Student's t -test are not fulfilled. We cannot, then, pool the estimates of the two variances, and an unmodified use of either the natural distribution or the t -distribution leads us to overestimate the significance of a difference between the two means.

Statistical methods that test the difference between means of unequal variance are usually based on the number of replicates and the degree of inequality of the variances. In an approximation (Bailey 1959, p. 51) that permits the use of a t -table, a reduced number of degrees of freedom is cal-

culated on the basis mentioned above. The formula of Bailey has been used in appropriate cases in the present thesis e.g. Table 2.9.

The above measures all serve to 'protect' the null hypothesis. On the other hand, the results may also allow us to increase the 'power' of the t-test, i.e. increase the probability of finding a difference when it actually exists. This can be done if the observations occur in pairs, usually by the same specimen or individual being treated in two different ways. Much of the biological variation is thus removed. Table 2.9 also gives an example of this procedure.

iv) Multiple comparisons

The essential problem of applying significance tests to more than two means is the number of possible comparisons. In general, for n means there are $n/2$ number of pairs, each of which can be compared $(n-1)$ times. Thus for 15 means there are 105 possible comparisons. The probability of wrongly rejecting the null hypothesis is the same for each comparison. Therefore, if we were to use Student's t-test repeatedly the joint probability of making a 'Type I error' would increase non-linearly with the number of means.

In the analysis of variance, which has been mentioned several times already, we test the null hypothesis that the samples are all from the same population. In some cases this may be sufficient for our purposes, e.g. in the question of combining results or in the regression analysis. However, in most cases we are still interested in knowing which of the means are significantly different, and the analysis of variance gives no decisions in this question. There may be good experimental reasons for using the t-test on a few preselected means, but for comparisons that are prompted by a posteriori inspection of the figures we need more conservative tests. There are many tests of multiple comparisons and a very strong subjective ingredient in the

choice between them. However, a discussion of the basic ideas may explain the rationale of the choice of method in the present thesis.

In multiple comparisons the level of significance (α) may either mean per comparison error rate ($= \text{Number of comparisons falsely declared significant} / \text{Total number of comparisons}$), or experimentwise error rate ($= \text{Number of experiments with at least one difference falsely declared significant} / \text{Total number of experiments}$). The relation between the corresponding protection levels, $(1-\alpha)$, is $(1-\alpha_E) = (1-\alpha_C)^k$, in which α_C and α_E represent per comparison and experimentwise error rates, respectively, and k is the number of comparisons (Steel, 1961). It follows from the expression that tests based on experimentwise error rates require an increasingly small α_C with increasing number of k 's in order to have a constant protection level. Duncan (1955), in presenting the multiple range test used in this thesis, points out that any increase in the protection level is made at the expense of losses in the power of a test. Therefore, the conservative tests based on constant protection levels will have a small probability of finding differences that are true.

The basic idea in Duncan's test is the analogy between a randomized block design that tests a number of independent differences simultaneously, and the same number of experiments necessary to test these differences separately. In the block design n means of randomized treatment combinations are necessary to test $(n-1)$ independent differences, but these could also have been tested in $(n-1)$ separate experiments. If $(n-1)$ null hypotheses are rejected in $(n-1)$ separate experiments then the chance of not rejecting the joint null hypothesis if all the null hypotheses are simultaneously true is $(1-\alpha)^{n-1}$. This joint protection level obviously becomes very small with an increasing number of experiments, but this is implicit in the protection level

chosen for each independent test. On the other hand, the chance of all null hypotheses being simultaneously true is a^{n-1} , which is much less, because a is a smaller number than $(1-a)$.

Duncan's tables are constructed to provide protection levels based on the degrees of freedom. This arises from a generalization of the analogy, mentioned earlier, between the block design containing n means, and the $(n-1)$ independent experiments. For n means the protection level is $(1-a)^{n-1}$. This is clearly a much higher protection level than would be obtained by applying t -tests to the $(n/2)(n-1) = k$ possible comparisons and obtaining a protection level of $(1-a_c)^k$, also mentioned earlier. Compared with the constant protection levels offered by the conservative tests it is much less if there are many means.

The crucial point in Duncan's test must be whether the analogy with the independent experiments is true to the extent that one can compare unknown a priori probabilities with the probabilities known after a number of independent tests.

Assumptions like these might critically affect the test if it were used alone. However, as Duncan's test is preceded by the analysis of variance in the present thesis, the joint null hypothesis has already been rejected and $(1-a_E)$ is known. In practice (Newman, 1975, personal communication) a number of various tests were applied to the Li^+ data of Table 4.5 (Scheffé, 1953; Duncan, 1955; Dunn, 1961; Dunnett, 1964, and the least-significant-difference (or L.S.D.) test as described e.g. by Parker, 1973). The results of Scheffé's test of all possible contrasts ($a_E = 0.1$), the L.S.D.-test ($a_c = 0.05$) and Duncan's test ($a = 0.05$) were the same placing the third value (2.200) in a position between two groups that were significantly different. Dunn's test using a predetermined number of contrasts ($a_E = 0.05$), and Dunnett's test using contrasts with a control gave essentially

the same result as above; the control value of Li^+ (2.060) was contrasted with the other four means and found significantly different from the values 2.247 and 2.265 in both tests, but not from any of the other values in any of the tests. The notation for the significance level in Duncan's test is without any suffix (C or E) because it cannot rightly be called, either per comparison error rate, or experimentwise error rate. This indicates the position of Duncan's test between liberal and conservative tests of multiple comparison. The outcome of their joint application to the same group of means is reassuring; it demonstrates in practice that the use of Duncan's test combined with the analysis of variance satisfy the requirements of power and protection.

5) THE INTERPRETATION OF RESULTS

Statistical methods permit us to draw quantitative conclusions in the presence of variation, but they are not concerned with the causes of variation, (Campbell, 1974, p. 1 - 2). We obtain a number telling us how likely it is that an event could have happened by chance, i.e. 'by absence of design or discoverable cause' (C.O.D., 1964), and we must then decide whether this likelihood is small enough for us to reject the null hypothesis. However, the subjective element becomes much stronger when we interpret the results which have been declared statistically significant.

An experiment in the biological sciences is a deliberate interference with ('treatment' of) a biological system. In a complex system such a treatment may trigger a number of events whose relation to the treatment is a very indirect one. Simplifying the system reduces the number of possible events caused by the treatment. However, this may also involve techniques that themselves induce uncontrolled effects altering the outcome of the treatment under investigation (Hillman, 1972). In both cases we are faced with many unin-

tentional ('confounded') effects whose elimination is decisive in the interpretation of the experiments. In fact, Section 1) and 2) of this chapter have been devoted mainly to the description of experiments on possible confounded effects. Often it is too time-consuming or outright impossible to eliminate confounded effects. However, there are statistical designs that allow us to control suspected confounded effects (Heath, 1970, p. 10).

Thus, by suitable precautions, we may decide which of the effects is responsible for the observed changes in the system, but this does not tell us how these changes were brought about, i.e. interpret the change in terms of a mechanism. It may assist our thinking to arrange biological systems of varying complexity and degree of interference in a hierarchy (Hillman, 1976). However, this may also result in an evaluation of the validity of experiments exclusively in terms of their 'naturalness'. There are various levels of interpretation according to the level of organization of the system under study. 'In general, the bigger the change of level from the experiment to the interpretation the more simplifying assumptions are needed and if the step is too large these become absurd' (Heath, 1970, p. 19).

In principle, experimental studies of a problem in biology should be applied to systems of diverse complexity in order to elucidate mechanisms, and their possible relevance to the intact organism in its natural environment; in practice, limitations in time and resources, both material and mental, may make such an approach very imperfect.

SUMMARY OF CHAPTER TWO

II 1A. Effects of isolation

1. The swelling, i.e. the difference between incubated and fresh wt., and the concentration of K^+ of the slices were used as easy and well established criteria of functional

activity.

2. Within the experimental error there was no significant effect of differences in delay from the killing of the animals till the incubation of the slices.

3. A review of the literature shows that the unavoidable cutting of the slices affects the tissue to considerable depth. As a consequence of differences in the functional activity between slices with one and two cut surfaces, only slices with one cut surface were used in the present experiments; a longitudinally vibrating monofilament nylon thread was used for the cutting.

4. The thickness of cerebral slices is restricted by the distance of diffusion of oxygen. Theoretical and practical considerations show that slices may profitably be cut somewhat thicker than originally calculated by Warburg.

II 1B. Effects of incubation

1. Evaporation, which may cause significant changes of the medium, was avoided by suitable precautions.

2. The pH of the unincubated media differed minimally between experiments. Evidence from the literature shows that small changes in the pH during incubation are of little consequence for the functional activity of the slices.

3. There was measurable leakage of K^+ from the slices to the medium. Though trivial under most circumstances, changes of the medium due to leakage of solutes may assume significance in some experiments.

4. Continuous agitation of the slices by the oxygen stream is important in order to ensure optimal functional activity. This was not always the case under the present experimental conditions and seems to be the only serious methodological error of the present experiments.

5. Probably due to this, the length of some of the experiments (Chapter Six) proved critical.

6. Homogenized cerebral tissue did not show any affinity towards Li^+ ; however, the method was insensitive.

7. Slices fixed in formol-saline were attempted as inert controls of metabolic effects, but proved unsuitable as they had a special affinity towards Li^+ and Na^+ .

II 2.The measurement of Li^+ , K^+ and Na^+

1. Recovery experiments with Li^+ showed that a satisfactory matrix matching of the standards was obtained to allow for all experimental conditions.

2. It was not possible to perform recovery experiments to check the measurements of K^+ and Na^+ in the tissue, but various control experiments ruled out serious systematic errors.

II 3.Calculation of the intracellular space

1. From a review of the literature it is concluded that so-called extracellular markers, if used with conventional techniques, are not reliable as indicators of the anatomical extracellular space in cerebral slices.

2. Fluid adhering to incubated slices makes up 2 to 15% of the weight according to reports in the literature. The magnitude of the figure depends on experimental circumstances, and on the method of determination and definition of adhering fluid.

3. Cerebral slices fixed in formol-saline were attempted as controls for extracellular fluid and adhering fluid, but were found unsuitable.

4. As a consequence of 1 - 3, measured amounts of Li^+ , K^+ and Na^+ in the slices are referred to incubated weight in the present thesis. This must be taken into account in the interpretation of kinetic experiments.

II 4.The use of statistics

The assumptions underlying the use of some common statistical tests are discussed. The conditions in biological experiments frequently call for conservative tests. The rationale of the present choice of statistical methods for multiple comparisons is explained.

II 5.The interpretation of results

In order to minimize unintentional effects and correlate the level of organization of the system under study with the level of interpretation, in principle, experimental studies of a problem in biology should be applied to systems of diverse complexity.

CHAPTER THREE

THE UPTAKE OF LOW CONCENTRATIONS OF Li^+ INTO CEREBRAL SLICES

The experiments of the present chapter and those of Chapter Four and Six were designed to investigate the kinetics of Li^+ in a composite cerebral tissue without the blood-brain barrier or other overriding mechanisms occurring in vivo and to use concentrations of Li^+ comparable to the serum levels of patients treated for manic-depressive disease.

The aim of the experiments of this chapter was to find out whether the influx was characterized by simple diffusion or facilitated diffusion, whether there was any indication of dependence on metabolism, and whether there might be evidence of heterogeneity of the cerebral cells towards the uptake of Li^+ .

METHODS

Preparation of tissue and incubation: Male Wistar albino rats weighing 200 - 250 g were killed by neck fracture. The brain was removed and one cerebral slice, weighing 40 - 80 mg was cut 0.5 mm in thickness from each hemisphere as described in the legend of Table 2.1.

They were pre-incubated for 30 min in a Krebs-Ringer bicarbonate glucose saline at 37°C , bubbled vigorously with a water saturated mixture of 95% O_2 : 5% CO_2 (or 95% N_2 : 5% CO_2 when anaerobic conditions were used). The incubating medium referred to in this thesis as 'standard' had the composition described in Table 2.2. The pH of the medium at room temperature was approx. 7.2. (Table 2.4).

Concentrated Li^+ solutions were added with a 50 μl constriction pipette after the pre-incubation. They were mixed rapidly by agitating the beakers; the time at which the mixing took place was used as the zero time for incubation. The incubation times for the initial uptake (min) were:

0.25; 0.50; 0.75; 1.0; 2.0; 3.0; 4.0 and 5.0.

Two slices were incubated in each beaker except where short incubation periods made this impossible. By this arrangement up to twenty slices could be used in each experiment. After incubation the slices were drained on a glass surface (McIlwain & Rodnight, 1962, page 116). They were weighed, and the incubated weights were used throughout to calculate the concentrations of the ions in the cerebral slices (Chapter 2.3 v)). The slices were also weighed routinely before incubation (Chapter 2.1 A iv)); the increase in weight during incubation was 15 - 20% in a standard medium.

The slices were homogenised by hand in 1 - 3.5 ml of 6% (w/v) trichloroacetic acid (TCA) depending upon the expected concentration of Li^+ .

An attempt was made to assess the relative contribution to the uptake of Li^+ during the first 15 s made by Li^+ in the adherent incubating media and by the diffusion of Li^+ into the extracellular space. For this purpose fixed slices were used. These were prepared as described in the legend of Table 2.9.

Measurement of Li^+ : Li^+ concentrations were measured with a Perkin-Elmer 303 atomic absorption spectrophotometer using an air-acetylene flame. The procedure and recovery experiments have been described in detail in Chapter 2.2. Li^+ was measured both in the slices and in the media. The latter measurements served as a check against gross errors of addition. The measured values were so close to those intended that the nominal values were used in all calculations (Table 2.3B).

Calculations: The ratio of concentration (R_t) of Li^+ between tissue and the medium is often used in the present thesis as

a convenient value, because it is independent of the concentrations if there is no saturation. In the calculations of the concentration ratio (R_C) between the cells and the medium in this and all other chapters, the following assumptions are made:

1. The specific gravity of the fluid in the intracellular space (ICS), extracellular space (ECS), and of the medium is one (Chapter 2.3i)).
2. The ratio of concentration between the ECS and the medium is one, i.e., unless specified, the ECS is considered to be in equilibrium with respect to Li^+ .
3. Li^+ is present exclusively in the ICS, ECS and in the medium.
4. The amount of solids is constant during the experiment, i.e., the mass of tissue and the volume of tissue water are proportional.

The assumed size of the ECS is always stated; usually the extremes of 20 and 40 μl per 100 mg incubated wt. and the notation '%' are used; if, for the sake of comparison with values in the literature, ECS is referred to fresh wt. this is always specified. As mentioned earlier (Chapter 2.3i)) the dry wt. (DW) of fresh rat brain is 18-19 mg per 100 mg (or %). An average swelling of 20% would reduce this figure to 15% of the incubated wt. (dw). In calculations the rounded figures of 20% for DW and 15% for dw have been used.

$$\text{Equations: (1) } (\text{ECS} + \text{ICS})\% = 100 - \text{dw}\%$$

$$(2) \quad 100 \times R_t = \text{ECS}\% \times 1 + \text{ICS}\% \times R_C$$

Hence R_C can be found. However, in the text, the shortened version:

$$(3) \quad R_C = (R_t - \text{ecs}) / \text{ics}$$

is used, using unit weight instead (see next paragraph).

Provided that assumptions (1)-(4) above are correct, then the dimensions in equation (1)-(3) can be reconciled, and the following units conjoined by or can be interchanged freely: (i) solids: mg per 100 mg tissue or % (ii) ICS, ECS, and medium: mg per 100 mg tissue or μ l per 100 mg tissue or % (iii) R_t : no units used (iv) R_c : no units used (v) ics and ecs: mg per mg tissue or μ l per mg tissue.

With a few exceptions, the initial uptake of each concentration of Li^+ into cerebral slices (Tables 3.1) was determined in three separate series at intervals from days to weeks in order to randomize adventitious effects. The straight lines were fitted by the method of least squares, sometimes after logarithmic transformation; the analysis of variance was used to test differences between the curves (Chapter 2.4 ii)). Unless otherwise indicated, the standard deviations of the straight lines were calculated as: $(\text{error variance}/S(x-\bar{x})^2)^{1/2}$, in which S is substituted for Sigma for typographical reasons.

Throughout the thesis, mean values have generally been shown with the number of digits required for the standard error of the mean to be shown with two digits (Bailey, 1959 p. 156).

Programmable calculators (Olivetti Programma (0) and Hewlett Packard, HP 25) were used for the calculations.

RESULTS

Initial uptake: The time course of the uptake of Li^+ into cerebral slices from an incubating medium 1.5mM in Li^+ is shown (Fig. 3.1). Besides 1.5mM Li^+ in the medium, five other concentrations of Li^+ from 0.5mM - 2.0 mM were examined (Table 3.1). The uptake from media 0.5mM in Li^+ was exceptional in that the influx measured in one experiment was significantly different from that of the two others, being less than half their value. As the regression line from this particular experiment was also much less significant than the regression

lines from the two corresponding experiments the results from the first experiment were discarded even though no obvious reason for the discrepancy could be found.

The combined regression lines, each representing one concentration of Li^+ in the medium and based on 16-36 values, had correlation coefficients from 0.93 - 0.97 (Table 3.1). Extrapolation of the lines to zero time showed concentrations, which were 11 - 17% of the corresponding concentrations in the media; the concentrations of Li^+ found in previously fixed slices incubated for 0.25 min were similar to these intercepts (Fig. 3.1). These intercepts were regarded as being due to Li^+ in adherent fluid and extracellular space (see Chapter 2.3 v)). Consequently, the slopes of the regression lines, obtained in the way described above, were used to express the unidirectional influx of Li^+ into cerebral slices (Table 3.1 column iii). Strictly speaking, flux should be stated as the number of moles crossing unit area in unit time (dimension: $\text{NL}^{-2}\text{T}^{-1}$). However, the area-to-volume relationship was not determined in the present experiments, and the cell area, which would be the relevant reference, is inaccessible to measurement. It is a reasonable assumption that the area-to-volume relationship is constant during the few minutes of uptake under standard conditions. The expression 'transfer' constant is used in the following about the corresponding rate constant with the dimension T^{-1} (Harris & Maizels, 1951, p. 507). In the present calculations of the transfer constants, the dry wt. and extracellular space of the slices have been disregarded. Again, these terms can be considered constant during the initial uptake.

The variance of the influx increased with its magnitude. This difficulty in the statistical analysis (Bartlett, 1937; Chapter 2.4 ii)) was obviated by comparing the transfer constants instead of the influxes (Table 3.1 column i, iii and v). The six estimates of the transfer constant were not significantly

different. The combined transfer constant was $(8.75 \pm 0.65) 10^{-2} \text{ min}^{-1}$ (mean $\pm$ S.D. of six estimates). The corresponding half-time ($T_{1/2}$) was 7.92 min.

Fractional equilibration for free diffusion into a plane sheet of thickness d , exposed to a solute on both sides can be expressed as follows:

$m_t/m_\infty = 1 - 8/\pi^2 (e^{-D\pi^2 t/d^2} + P_n)$ where m_t/m_∞ is a fraction relating the amount of transported solute after t units of time (m_t) to the amount present when full equilibrium has been reached (m_∞). D is the diffusion coefficient; P_n represents the second and subsequent exponential terms in a rapidly converging series; these terms are small enough to be ignored for the present purposes (Kotyk & Janáček, 1975 p. 37 eq (24a), notations slightly modified). Disregarding P_n , rearranging, and taking logarithms, the expression simplifies to $T_{1/2} = 0.05 d^2/D$ at half equilibration. The diffusion constant for Li^+ , measured as LiCl at 25° between 0.6 mM and 2.3 mM is $1.34 \times 10^{-5} \text{ cm}^2/\text{s}$ (Harned & Hildreth, 1951, Table II, column 2, mean of 3 values). With a slice thickness of 0.5 cm, this would give a half-time of free diffusion of 9 s. However, in a cerebral slice, the presence of cells would increase the mean diffusion path. Therefore, the half-time of exchange between the medium and the extracellular space of the slices must be multiplied by a correction factor, λ^2 . The correction factor is dependent upon the size of the extracellular space in the following way: $\lambda = 1 + 3 V_c/V_t (1 - \pi/4)$, where V_c is the volume of (spherical) cells, including solids, and V_t is the total volume of the slice (McLennan, 1957, eq 9). With an extracellular space of 20% or 40%, this would result in half-times of 21 s or 17 s, respectively.

Net-uptake: A steady state in the uptake was seen after approx. 1 h incubation with 0.5 mM, 1.0 mM and 1.5 mM Li^+ in the medium. The results of these studies of the net uptake were combined, as the transfer constants of the unidirectional

influx were not significantly different. In order that the results could be combined, all concentrations measured during the uptake were calculated as a percentage of their concentrations at steady state. An inverse semilogarithmic plot of the mean values is shown (Fig. 3.2, closed circles). Apart from the initial phase, the points were naturally distributed, suggesting a single monoexponential function. Even if the values measured before 1 min were included in the plot, the experimental scatter was too large to allow calculation of the fast (extracellular) component, which must be there. The apparent transfer constant of the net uptake was $(5.58 \pm 1.63) 10^{-2} \text{ min}^{-1}$ (mean $\pm$ S.D. of 3 estimates, 169 slices), corresponding to a half-time of 12.4 min.

The net uptake in the absence of oxygen was measured with 0.5 mM and 1.5 mM Li^+ in the medium, calculated and plotted in the same way (Fig. 3.2, open circles). The apparent transfer constant of the net uptake was $(1.862 \pm 0.007) 10^{-2} \text{ min}^{-1}$ (mean $\pm$ S.D. of 2 estimates, 36 slices) corresponding to a half-time of 37.3 min.

DISCUSSION

In the choice of incubation times for the determination of unidirectional influx with the present techniques, there were three considerations, first, exclusion of interference from diffusion through the extracellular space, secondly, exclusion of back diffusion, and thirdly, inclusion of enough samples and sufficiently long intervals of incubation times for statistical significance of the regression analysis.

1) An approximate equilibrium would be obtained after 4-5 half-times. Using the corrected values of 21 s and 17 s for extracellular diffusion of Li^+ , calculated earlier, this would take more than a minute even with a high value of the extracellular space.

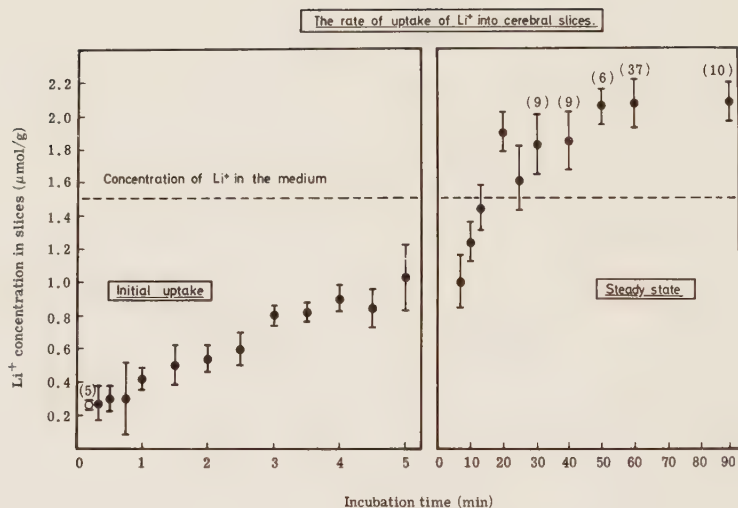


FIG. 3.1

Initial uptake, net transfer and steady state of Li^+ in cerebral slices. The slices were incubated for the times indicated in a standard medium also containing Li^+ 1.5 mM. The values are the means $\pm$ S.D. of 3 slices, except where (n) is shown in brackets. Five slices, which had previously been fixed in formol-saline and rinsed, 0, were incubated in the above medium for 15 s (see Methods). For graphical reasons the initial uptake (left side) is shown expanded relative to the right side. The regression line for the initial uptake, $y = bx + a$, gave values of $b = 0.151 \text{ mmol}/(\text{kg min})$, and $a = 0.260 \text{ mmol}/\text{kg}$.

TABLE 3.1 INITIAL UPTAKE OF Li^+ INTO CEREBRAL SLICES

(i) Li^+ in medium (mM)	(ii) number of slices	(iii) influx of Li^+ ($\mu\text{mol}/(\text{kg min})$)	(iv) correlation coefficient	(v) transfer constant (10^{-2} min^{-1})
0.50	16	46.8 ± 3.3	0.97	$9.36 \pm .67$
0.75	24	60.7 ± 4.9	0.94	$8.09 \pm .66$
1.00	23	79.8 ± 6.2	0.95	$7.98 \pm .62$
1.25	24	109.0 ± 7.2	0.96	$8.72 \pm .58$
1.50	36	150.7 ± 10.6	0.93	$10.05 \pm .71$
2.00	23	165.4 ± 11.7	0.96	$8.27 \pm .59$

The uptake was measured at various intervals during the first five min (see Methods). The figures in columns (iii) and (v) are the slopes $\pm$ S.D. calculated from the number of slices indicated in column (ii). The transfer constants were calculated by dividing the values in column (iii) by the corresponding values in column (i).

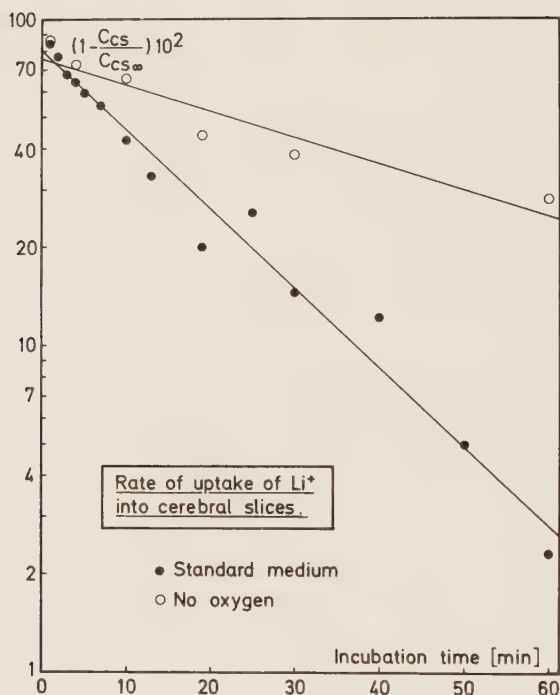


FIG. 3.2

The effect of anoxia on the net transfer of Li^+ . The slices were incubated for the times indicated in standard media also containing Li^+ 0.5 mM, 1.0 mM or 1.5 mM. In order to combine the results, the concentrations in the cerebral slices (C_{cs}) were related to the corresponding values at steady state ($C_{cs\infty} = 0.686 \text{ mmol/kg}$, 1.384 mmol/kg or 2.096 mmol/kg , respectively, obtained after 90 min incubation). Each of the calculated means, ●, represents 6 to 40 slices. The data (Fig. 3.1) were included in this plot. Other slices were incubated in the absence of oxygen in standard media containing Li^+ 0.5 or 1.5 mM. The mean values were calculated as above, using $C_{cs\infty} = 0.686 \text{ mmol/kg}$ and 2.096 mmol/kg as the steady state concentrations. The mean values, ○, represent 6 slices each. In the equation, $y = (1 - ae^{-kt})$ the following values were found: $a = 79.9 \times 10^{-2}$ and 75.2×10^{-2} ; $k = 5.58 \times 10^{-2} \text{ min}^{-1}$ and $1.86 \times 10^{-2} \text{ min}^{-1}$ in the presence and absence of oxygen, respectively.

2) The requirement of no efflux during the measurement of unidirectional influx is obviously impossible to fulfil, because the concentration in the tissue has to be measurable and increasing during the incubation period in order to estimate influx. An average concentration in the tissue of 40% of the concentration at steady state is found from the expression $(1 - e^{-kt}) 10^2$, using the intercept and net transfer constant calculated above (legend of Fig. 3.2), and $t = 5$ min. With a concentration ratio $C_{cs\infty}/C_{\text{medium}}$ of 1.5, this is 60% of the concentration in the medium. The corresponding value in the cells would be higher or lower than that, depending on the size of the extracellular space (62% and 44% if the extracellular space is 20% and 40% respectively, and the dry wt. is 15%). The relative importance of the intracellular concentration would depend on the rate of efflux, which was determined later (Chapter Six).

3) Theoretically, the initial uptake would have to exhibit zero-order kinetics if it were to express unidirectional influx. The regression analysis showed highly significant linear relationships (Table 3.1, column iv). However, as discussed above, there must be systematic deviations at both ends of the curves. In practice, experimental error seems to hide such deviations (Fig. 3.1). Among other things, the statistical significance of a linear relationship depends on $(x_i - \bar{x})$, the deviations of the independent variables from their mean value. Therefore, a shortening of the observation period to e.g. 1 - 4 min., though theoretically correct, might make the observed rates non-significant in the presence of the random error inherent in this kind of experiments. This was often found to be the case in the experiments of Chapter Five.

Systematic deviations in the initial uptake after short and long incubation times would tend to oppose each other. Extrapolations to zero incubation time of the kinetic curves give a rough estimate of the amount of Li^+ in the extracellular

space and adhering fluid, as was mentioned earlier. There are two kinds of systematic errors in this estimate, a) an inevitable error dependent on the size of the spaces, and the difference between the rate constants of the free diffusion and the membrane limited diffusion; this error can be corrected (Huxley, 1960) and will be dealt with in Chapter Six; b) an avoidable error depending on the choice of incubation times; this was pointed out in 1) and 2) above. If the measured values are removed from both extremes of the kinetic curves, the resulting change of the intercept will show which of the opposing tendencies in 1) and 2) are the strongest. The mean value of the six intercepts corresponded to 13% of the concentrations in the media. In the experiments of Chapter Five, in which measurements took place from 1 min to 4 min, the mean of eight estimates gave 18%. These results indicate that extracellular diffusion rather than back diffusion from the cells influence the results. However, the effect was not great. Correction of the initial uptake by removal of the values measured before 1 min reduced the transfer constants (Table 3.1) by approx. 5%.

Disregard of the presence of solids and extracellular space of the tissue results in an unknown underestimation of the transfer constants, but would not affect the linearity.

The findings in fixed slices incubated for 15 s in the presence of Li^+ are discussed in Chapter 2.3 iv). Their similarity with the intercept is probably a coincidence.

Whereas the values of the rate constants stated (Table 3.1) may be inaccurate, there is no doubt that they represent, primarily, membrane limited diffusion, as they are no more than one twentieth of the rate constants for free diffusion into the extracellular space of cerebral slices.

It is not possible to conclude from the present kinetic experiments whether the uptake was passive or carrier mediated because of the relatively narrow concentration area. Satura-

tion was not seen within the range of concentrations of Li^+ employed. If the transport system, or part of it, is saturable, it must either be completely saturated at very low concentrations, or the apparent K_m must be well above the concentrations used clinically.

The plot of the net uptake from 1 min to 60 min was compatible with one compartmental first order kinetics. As mentioned earlier, this does not exclude an extracellular component, but it would be difficult to demonstrate with the experimental arrangement of the present thesis. There was no indication of more than one cellular component. Therefore, within the experimental error, the various types of cells in a cerebral slice cannot be distinguished in their kinetics of uptake towards Li^+ .

Recently, the data of the net transfer using 1.5 mM Li^+ in the medium (Fig. 3.1; Fig. 1 in Wraae et al., 1976) were replotted to fit Fick's diffusion law (Pradhan et al., 1977; Fig. 2b). On the basis of this replot, the authors claimed that they could demonstrate multiple local steady states. The semilogarithmic plot (Fig. 3.2, closed circles) is based on the same transformation of data as that of Pradhan et al.; it differs from the corresponding plot of the data (Fig. 3.1, Li^+ 1.5 mM) only in the influence on the points of the additional data (0.5 mM and 1.0 mM Li^+ in the medium). Clearly, the authors have only used some of the data, but no selection criteria were stated, nor was there any evidence of statistical treatment.

In the absence of oxygen, the rate of uptake of Li^+ was about one third of the normal uptake (Fig. 3.2, open circles). This demonstrates that the uptake is dependent on metabolism, but is not necessarily evidence of active uptake, because the effect may be due to changes of electrochemical potentials or transport systems used in exchange diffusion. The plot showed some deviation from linearity. It was noted that the

swelling of the tissue was dependent on the incubation time, demonstrating that changes in the tissue continued after 30 min pre-incubation. Thus the assumption of the constant area-to-volume relationship during the uptake is unlikely to be true, and the non-linearity is probably due to continuous changes in the tissue.

SUMMARY

1. The influx (initial uptake, 15 s to 5 min) of low concentrations (0.5 - 2 mM) of Li^+ showed no evidence of saturation. The half-time of the influx was 7.9 min compared with a calculated half-time of 17 - 21 s for free diffusion in the extracellular space of the slices.
2. The value of the influx was influenced both by diffusion of Li^+ through the extracellular space and back diffusion, but these opposing influences do not invalidate its interpretation as membrane limited uptake.
3. The net uptake of Li^+ proceeded to a steady state after 1 h. incubation. A semilogarithmic plot was compatible with one compartmental first order kinetics. In the absence of oxygen, the rate of uptake was about one third of the normal uptake.

CHAPTER FOUR

Li⁺ IN CEREBRAL SLICES AT STEADY STATE. THE INTERACTION OF LOW CONCENTRATIONS WITH OTHER CATIONS

The experiments of Chapter Three demonstrated that the uptake of Li⁺ in cerebral slices was concentrative and that its rate was dependent of the oxygenation of the slices.

The aim of the following experiments was to examine the interactions of Li⁺ with K⁺, Na⁺, Ca²⁺ and Mg²⁺ using concentration ranges of these ions embracing those found in the extracellular fluid of patients.

METHODS

Preparation of tissue and incubation: Cerebral slices were prepared as described in Chapter Three.

They were pre-incubated for 30 min in the absence of Li⁺ and, unless specified, in the presence of 1.5 mM Li⁺ for 1 h. As described earlier, concentrated Li⁺ solutions were added with constriction pipettes so that transfer of the slices during the 1.5 h of incubation was unnecessary. The composition of the standard medium (Table 2.2) was altered as described in RESULTS (Fig. 4.2 - 4.5). The concentration of NaCl was adjusted to keep the osmolarity constant in the experiments in which the effects of different concentrations of K⁺, Ca²⁺ and Mg²⁺ were studied. Choline⁺ was not used to replace Na⁺ because it has been shown to be taken up from low concentrations of the medium by saturation kinetics in cerebral slices (Schubert *et al.*, 1966).

Measurement of cations: The measurements of Li⁺, Na⁺ and K⁺ are described in Chapter 2.2. The concentrations of Ca²⁺

and Mg^{2+} in the fresh media were checked on a few occasions during the whole series of experiments and found to agree with the expected values. These analyses were kindly performed by the Department of Clinical Biochemistry, University of Surrey.

Calculations: The experiments (Fig. 4.2 - 4.5) were carried out in three separate series at intervals from days to weeks in order to randomize adventitious effects. It was planned to use four slices for each of the five concentrations in the medium in each of the latter experiments, but technical mistakes reduced the number to two on three occasions (Fig. 4.3 - 4.4). Also, in addition to the five concentrations of K^+ already investigated, the effects of two more concentrations of K^+ in the medium were studied subsequently in three further series of similar experiments (Fig. 4.2).

There was some evidence of variation between experiments made on different days. The means of replicates from each experiment were used for the statistical analysis, giving an appropriate reduction in the degrees of freedom to take this into account. With this arrangement the inconsistencies introduced by the missing values and additional experiments could be ignored. The analysis of the results from the experiments in which Mg^{2+} in the medium was varied was based on the total number of slices, since there was an equal number of replicates (Table 4.5).

The analysis of variance was used to examine the means of a combined experiment. If a significant variation was found, the statistical analysis was proceeded with in the following manner: a straight line was fitted to the results if, both the linear relationship between the independent variable and the treatment means was significant, and any non-linear component in the relationship was not significant (Bliss, 1967 p. 422 - 424). If, using the above criteria, the data were

not adequately represented by a straight line, Duncan's multiple range test was used to obtain sub-groups, in each of which the members were not significantly different (Duncan, 1955). These procedures were discussed in Chapter 2.4, ii) & iv).

The standard deviations shown in Fig. 4.2 - 4.5 were calculated from 12 replicates unless otherwise indicated. They were not used in the statistical analyses and are included to represent the overall variation of the results obtained.

RESULTS

Steady state concentrations and their dependence on metabolism: The steady state ratio of concentrations of Li^+ in the tissue relative to the medium was found to be 1.659 ± 0.035 (the slope of the regression line $\pm$ S.D. of 20 samples), and appeared to be constant at concentrations from 0.5 mM - 1.5 mM Li^+ in the medium, as the correlation coefficient was 0.996 (Fig. 4.1). In the absence of glucose or oxygen in the incubating medium the uptake of Li^+ was significantly lower, and the ratio of concentration of Li^+ between the tissue and the medium approached unity (Fig. 4.1). The swelling was 40 - 60% in the absence of glucose or oxygen. If the fluid taken up has the same composition as the medium (Leaf, 1956), it can be calculated that correction for the increased swelling would raise the values for Li^+ in the slices by a maximum of 2.5%. This would not affect the significance of the differences, and therefore the corrections are not shown.

The effects of Li^+ on the K^+ , Na^+ , and swelling of the slices: In the experiments mentioned above (Fig. 4.1) the K^+ , Na^+ and swelling of the slices were also recorded. Within this range of concentrations none of the results were significantly different (Table 4.1).

Values for the K^+ , Na^+ , and the swelling of slices incubated

for 1 h in a standard medium without Li^+ were compared with those from all the experiments in which slices were incubated under the same conditions in the presence of 1.5 mM Li^+ . In the absence of Li^+ in the medium the concentration of K^+ was higher, the concentration of Na^+ was lower, and the swelling slightly less pronounced, than in the presence of Li^+ . The results are shown (Table 4.2); the absence of statistical significance of these differences is discussed in Chapter 2.4i).

The influence of K^+ , Na^+ , Ca^{2+} and Mg^{2+} in the incubating medium on the steady state concentration of Li^+ in the cerebral slices

Potassium ions (Fig. 4.2): The concentration of Li^+ in cerebral slices after 1 h incubation increased in a non-linear way as the concentration of K^+ in the incubating medium decreased. In the absence of K^+ the uptake of Li^+ was significantly higher than in the presence of any of the concentrations of K^+ that were used in the medium, and it was approx. 50% higher than the uptake in the presence of 5 mM K^+ in the medium. The statistical significance of the differences between the uptake of Li^+ in the presence of various concentrations of K^+ in the medium was assessed by a multiple comparison test, and the results are shown (Table 4.3).

The concentration of K^+ in the slices decreased, and the concentration of Na^+ increased, both in a non-linear way, when the concentration of K^+ in the medium was lowered. In the absence of K^+ in the medium the concentration of K^+ in the slices was approx. 30 mM and that of Na^+ was approx. 125 mM. The swelling of the slices was maximal (26%) in the absence of K^+ in the medium, and minimal within a physiological range of concentrations (Table 4.3).

In two experiments ouabain (10^{-5}M) was added to the media (not shown). This caused the slices to loose K^+ and gain Na^+ so that each of their concentrations were approx. equal to those of the media after 1 h incubation. The Li^+ of the slices also fell to values very near those of the media. The

swelling of the slices was even more pronounced than in the absence of oxygen.

Sodium ions (Fig. 4.3): There was a highly significant linear relationship between the concentration of Na^+ in the tissue and in the medium (120 mM - 170 mM). The regression between the two was found to be 0.80 with a correlation coefficient of 0.80 ($n = 15$). The Li^+ , K^+ and the swelling of the slices were not affected significantly by these changes in the concentration of Na^+ in the medium.

Calcium ions (Fig. 4.4): There was a highly significant inverse linear relationship between the concentration of Li^+ in the slices and the concentration of Ca^{2+} in the medium; the slope was $-0.13 \text{ mM Li}^+ \text{ in the tissue/mM Ca}^{2+} \text{ in the medium}$, and the correlation coefficient was 0.83 ($n=15$). The concentration of K^+ in the tissue relative to the Ca^{2+} concentration in the medium also showed a highly significant relationship; the slope was $3.9 \text{ mM K}^+ \text{ in the tissue/mM Ca}^{2+} \text{ in the medium}$, and the correlation coefficient was 0.66 ($n=15$). The concentration of Na^+ in the slices was approx. 120 mM, and the swelling was approx. 28% in the absence of Ca^{2+} in the medium. Each of these values was significantly higher than those found in the presence of Ca^{2+} (Table 4.4).

Magnesium ions (Fig. 4.5): The concentration of Li^+ in cerebral slices was approx. 10% higher when the Mg^{2+} concentration in the medium was increased from 1.3 mM to 2.0 and 2.6 mM; this difference was significant (Table 4.5). The concentration of K^+ in the slices was approx. 20% higher when the Mg^{2+} concentration in the medium was increased as above; this difference was also significant (Table 4.5).

There was a significant inverse linear relationship between the concentration of Na^+ in the slices and the concentration of Mg^{2+} in the medium; the slope was $-3.8 \text{ mM Na}^+ \text{ in the tissue/mM Mg}^{2+} \text{ in the medium}$, and the correlation coefficient was 0.61 ($n = 15$). The swelling of the cerebral slices

was not significantly altered by changes in the concentration of Mg^{2+} in the medium.

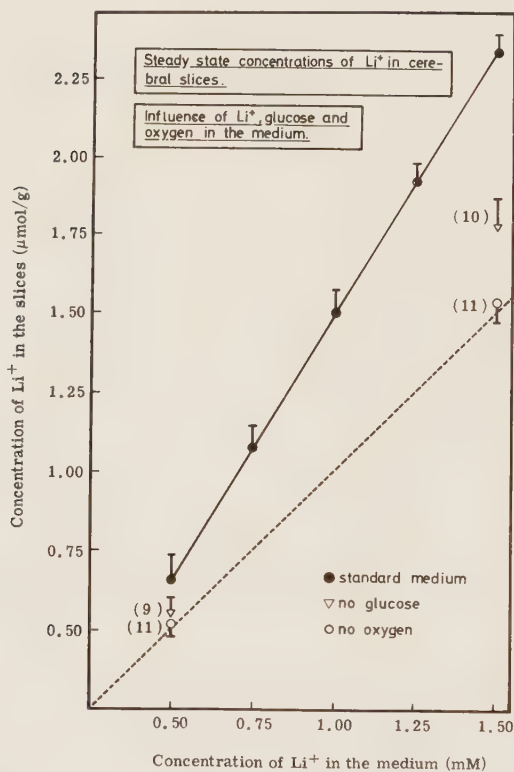


FIG. 4.1

Slices were incubated for 1 h in standard media also containing Li^+ in the concentrations indicated. Each of the filled circles represents the mean $\pm$ S.D. of 4 slices. Other slices were incubated in the absence of glucose or oxygen in standard media containing Li^+ 0.5 or 1.5 mM. These values are the means $\pm$ S.D. of (n) shown in brackets. The broken line indicates the 45° slope.

TABLE 4.1 INFLUENCE OF THE Li^+ CONCENTRATION ON THE K^+ , Na^+ AND THE SWELLING OF CEREBRAL SLICES

Li^+ in medium (mM)	0.5	0.75	1.0	1.25	1.5
K^+ in slices (mmol/kg)	65.6 ± 9.4	68.2 ± 5.4	68.4 ± 3.4	69.6 ± 3.2	70.7 ± 3.1
Na^+ in slices (mmol/kg)	92.4 ± 6.1	94.0 ± 6.9	94.8 ± 7.4	93.3 ± 9.5	92.3 ± 7.3
swelling (% of fresh wt.)	17.7 ± 5.0	17.8 ± 2.7	15.7 ± 4.7	14.1 ± 2.7	13.5 ± 2.8

Slices were incubated for 1 h in a standard medium also containing Li^+ in the concentrations indicated. Each value is the mean $\pm$ S.D. for four slices. The slices are identical with those of Fig. 4.1. None of the values were significantly different by the analysis of variance.

TABLE 4.2 THE EFFECT OF Li^+ IN THE INCUBATING MEDIUM ON THE CONCENTRATIONS OF K^+ , Na^+ , AND THE SWELLING OF CEREBRAL SLICES

	(a) No. of experiments	(b) K^+ in slices (mmol/kg)	(c) Na^+ in slices (mmol/kg)	(d) K^+ and Na^+ (mmol/kg)	(e) swelling (% of fresh wt.)
Li^+ not added	4 (28)	63.8 ± 15.8	96.2 ± 8.8	160.0 ± 7.8	15.2 ± 10.1
Li^+ (1.5 mM)	9 (37)	56.2 ± 15.3	101.0 ± 10.6	157.2 ± 10.2	18.9 ± 8.1
significance of differences (P values)		n.s	n.s	n.s	n.s

Cerebral slices were incubated for 1 h in a standard medium in the presence or absence of LiCl (1.5 mM). Values presented are the means $\pm$ S.D. of the no. of experiments indicated in column (a). The corresponding no. of slices is shown in brackets. Values in column (d) were obtained by addition of individual values in columns (b) and (c). Student's t-test was used to evaluate the significance of the differences. For a discussion of the statistics : See Chapter 2.4 i.

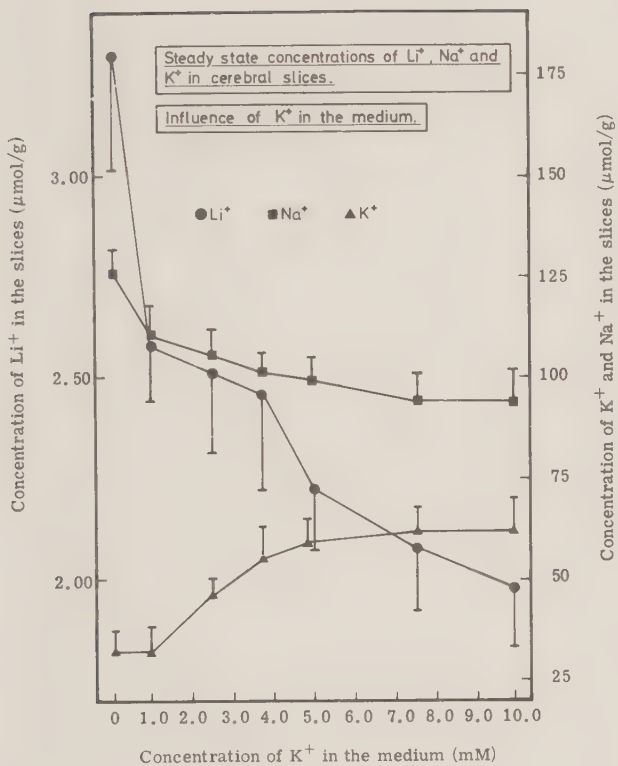


FIG. 4.2

Cerebral slices were incubated for 1 h in media containing 1.5 mM Li^+ and the concentrations of K^+ indicated. The values are the means $\pm$ S.D. of 12 slices.

TABLE 4.3

STATISTICAL ANALYSIS OF RESULTS IN FIG 4.2

(a) CONCENTRATIONS OF CATIONS IN THE SLICES

	0.0	1.0	K ⁺ in medium (mM)				7.5	10.0	no. of means	overall significance (P values)	overall S.E.M.
			2.5	3.7	4.9						
Li ⁺ (mmol/kg)	3.303	2.578	2.514	2.460	2.216	2.076	1.977		21	< 0.001	0.101
K ⁺ (mmol/kg)	32.2	31.6	45.7	54.9	59.3	62.4	62.2		21	< 0.001	2.66
Na ⁺ (mmol/kg)	125.9	109.9	106.3	101.3	99.2	94.2	94.0		21	< 0.001	2.54

(b) SWELLING OF THE SLICES

	0.0	1.0	K ⁺ in medium (mM)				3.7	4.9		
			10.0	7.5	2.5					
swelling (% of fresh wt.)	26.1	24.5	21.7	18.1	19.0	16.8	15.4		21	< 0.01
										1.35

A multiple range test for 5% significance was used (Duncan, 1955). In this test, any two means not underscored by the same line are significantly different; any two means underscored by the same line are not significantly different. Sometimes it has been necessary to rearrange the values in the tables in order to accommodate this convention. For reasons explained in Methods, the analysis of variance preceding Duncan's test was based on the reduced no. of values indicated in the table. The S.E.M. used in Duncan's test was calculated as (error variance/3)^{1/2}.

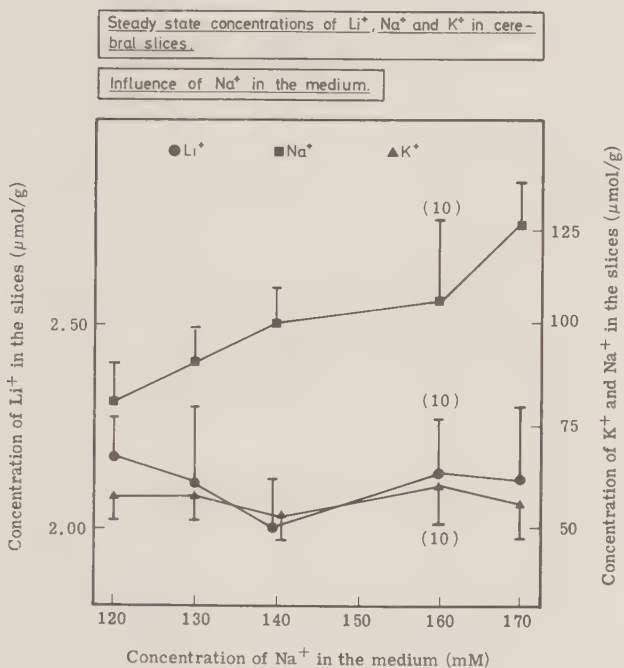


FIG. 4.3

Cerebral slices were incubated for 1 h in media containing 1.5 mM Li^+ and the concentrations of Na^+ indicated. The values are the means $\pm$ S.D. of 12 slices except where (n) is shown in brackets.

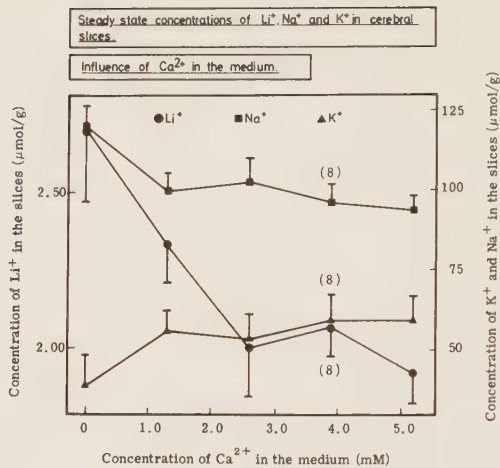


FIG. 4.4

Cerebral slices were incubated for 1 h in media containing 1.5 mM Li^+ and the concentrations of Ca^{2+} indicated. The values are the means $\pm$ S.D. of 12 slices, except where (n) is shown in brackets.

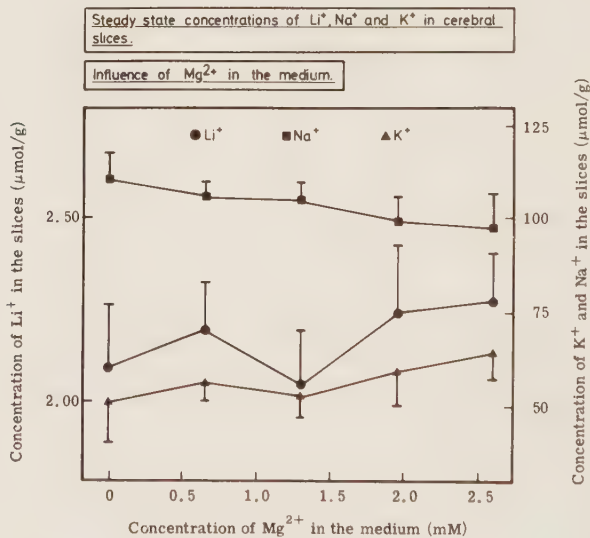


FIG. 4.5

Cerebral slices were incubated for 1 h in media containing 1.5 mM Li^+ and the concentrations of Mg^{2+} indicated. The values are the means $\pm$ S.D. of 12 slices.

TABLE 4.4

STATISTICAL ANALYSIS OF RESULTS IN FIG. 4.4

CONCENTRATION OF Na⁺ AND SWELLING OF SLICES

	Ca ²⁺ in medium (mM)			no. of means	overall significance (P values)	overall S.E.M.
	0.0	1.3	2.6	3.9	5.2	
Na ⁺ (mmol/kg)	<u>119.1</u>	<u>99.4</u>	<u>102.4</u>	<u>96.0</u>	<u>92.8</u>	3.02
swelling (% of fresh wt.)	<u>27.9</u>	<u>18.1</u>	<u>19.8</u>	<u>18.5</u>	<u>17.0</u>	1.40

A multiple range test for 5% significance. The S.E.M. used was calculated as $(\text{error variance}/3)^{\frac{1}{2}}$. The analysis of variance preceding this test was based on the reduced no. of values indicated in the table (see Methods). Further details of the statistical test are given in the legend of Table 4.3.

TABLE 4.5
 STATISTICAL ANALYSIS OF RESULTS IN FIG. 4.5.
 CONCENTRATIONS OF Li^+ AND K^+ IN THE SLICES.

	Mg^{2+} in medium (mM)			no. of slices	overall significance (p values)	overall S.E.M.
	1.3	0.0	0.95	1.95	2.60	
Li^+ (mmol/kg)	<u>2.060</u>	2.096	<u>2.200</u>	2.247	2.265	0.047
K^+ (mmol/kg)	<u>50.5</u>	<u>51.1</u>	<u>56.1</u>	<u>59.2</u>	<u>63.6</u>	1.88

A multiple range test for 5% significance. The S.E.M. used was calculated as $(\text{error variance}/12)^{\frac{1}{2}}$. The analysis of variance preceding this test was based on the total number of slices, as indicated in the table (see Methods). Further details of the statistical test are given in the legend of Table 4.3.

DISCUSSION

Steady state concentrations in the standard media: The concentration ratio for Li^+ between the tissue and medium in standard media was found to be 1.3 - 1.7. Kjeldsen et al. (1973), using the same techniques, found the slightly higher ratio of 1.9. This difference might partly be due to the Ca^{2+} concentration of 1.5 mM used in the incubation medium of these authors, as there was an inverse relationship between Li^+ and Ca^{2+} (Fig. 4.4). In a study on rat brain (Christensen, 1974), Li^+ was found to accumulate in the particulate fraction of the homogenate after having been administered in the food of the rats for 2 - 3 months. Addition of Li^+ to cerebral homogenates in vitro resulted in an accumulation ratio that was lower than in vivo even after 24 h incubation, but nevertheless above one. Entrapment of free Li^+ within semi-intact neurons was considered unlikely, but not controlled for. Redistribution during centrifugation was not considered. The dependence of the cerebral concentrations of Li^+ on metabolism is evident (Fig. 4.1). However, this in itself does not justify any conclusions about active uptake (Wraae et al., 1976) as was discussed in the previous chapter. In living rats with stable serum concentrations Li^+ is concentrated in the brain relative to the cerebrospinal fluid; this is not due to active removal of Li^+ from the cerebrospinal fluid (Chapter Seven). Therefore the ability to concentrate Li^+ must be a property of the cerebral tissue itself.

The effects of Li^+ on the K^+ and Na^+ of the slices: In the present experiments (Table 4.1 & 4.2) as well as experiments to be described later (Table 6.1), there was no statistically significant relation between the Li^+ in the medium and the K^+ and Na^+ of the slices. Kjeldsen et al. (1973) found that the presence of approx. 2 mM Li^+ in their standard medium caused a significant decrease of K^+ of almost 10 mmol/kg in

the slices. A direct demonstration of the effects of low concentrations of Li^+ in the medium on the uptake of K^+ into cerebral slices was reported by Israel et al. (1966); they measured the K^+ uptake of slices during recovery from anoxia; after 20 min re-oxygenation, the K^+ uptake could be seen to be dependent on the concentration of Li^+ in the medium in the range of 1 mM to 10 mM. Bianchi et al. (1977) using Na^+ loaded sciatic nerve investigated the effects of Li^+ on the Na-pump in the absence and presence of K^+ ; when K^+ was restored to the medium the uptake of K^+ was higher in the presence of 2 mM Li^+ than in its absence. No doubt, the effects of total anoxia (e.g. Table 2.6b) are more profound than inhibition of the Na - pump caused by the absence of K^+ so that the experiments of Israel et al. and those of Bianchi et al. may not be comparable.

The absolute difference between the K^+ of the slices in the absence and presence of Li^+ was approx. the same in the present experiments (Table 4.2) and those of Kjeldsen et al., but critical re-examination of the statistics (Chapter 2.4) leaves some doubt about the significance of the results. The data of Kjeldsen et al., Table 1, cannot be assessed in a similar way. Nevertheless, there may well be a real difference between the present experiments and theirs. First, there was no proportionality between the concentration of Li^+ in the medium and the K^+ of the slices (Table 4.1); secondly, the finding could not be reproduced in a later experiment (Table 6.1); thirdly, the Na^+ and swelling showed an increase in the presence of Li^+ in Table 4.2, suggesting a decrease in the functional state of the slices, whereas the Na^+ was lower and the swelling was unaffected by Li^+ in the experiments of Kjeldsen et al. As was discussed earlier (Chapter 2.1 Bii) the concentrations of K^+ in the slices from the experiments of the present thesis were generally lower than those published by others using the apparatus of Franck et al. (1968); the values also seem somewhat less

constant (e.g. Table 4.1 & 6.1). Therefore, it may well be that differences in the functional state of the slices could explain the differences between the results of Kjeldsen et al. and those of Table 4.2.

Effects of other cations in the medium: Li^+ transport in red cells has been studied intensively during the last few years and can be considered well characterized (see Chapter Eight). As summarized by Greil & Eisenried (1978), there are at least three components in the Li^+ uptake of red cells: 1) Li^+ uptake due to the action of the Na-pump, 2) uptake mediated by the Na-dependent Li^+ countertransport system, 3) uptake due to 'leak diffusion'. This can be generalized into the following scheme, which should be valid for the transfer of cations irrespective of tissue and species: 1) well defined systems of active transport, 2) ion exchange systems, possibly with an actively transported ion, 3) passive transport ('leak diffusion'), 4) binding to subcellular structures, 5) a concentration dependent combination of the possibilities mentioned above, 6) a transport system that has not been characterized previously. High concentrations of Li^+ have frequently been used in the experiments of the papers to be discussed. Although this would seem to render the results less relevant from a clinical point of view, such experiments are often necessary in order to characterize transport mechanisms that may also operate at low concentrations of Li^+ .

Potassium ions: 1) It is not surprising that many authors have claimed to observe effects of Li^+ on the K^+ site of the Na-K-pump or the corresponding ATP-ase, as this is the best characterized transport system. For a review see Skou (1965). Kjeldsen et al. (1973) suggested that Li^+ in addition to activating the K^+ site of the Na-K-ATPase might inhibit linked uptake of K^+ and Cl^- . However, their effects of Li^+ on the Na^+ seems to be statistically significant only at a K^+ concentration in the medium above 10 mM (l.c., Fig.1) and the only

Cl^- measurements shown in the absence and presence of Li^+ are not significantly different (l.c. Table 1), so that this hypothesis is not supported strongly by their own data. Ploeger & Hertog (1973), recording potentials in the desheathed vagus of the rat, found that the potassium activated response decreased in the presence of Li^+ 1-5 mM and could demonstrate competition between K^+ and Li^+ (l.c. Fig. 3 & 4). Smith (1974) using frog skeletal muscle incubated in a 5 mM Li^+ Ringer solution observed a ouabain sensitive, K^+ competitive uptake of Li^+ , which was up to half of the Li^+ influx. Haugaard et al. (1975) in isolated rat diaphragm found that the concentration of Li^+ in the tissue in the presence of 25 mM Li^+ in the medium increased by approx. half when K^+ was absent from the medium (l.c. Table 3). In the experiments by Bianchi et al., mentioned earlier, weak stimulation of the Na-pump was seen in the presence of 10 mM Li^+ when K^+ was absent from the medium. Beaugé & Ortiz (1972) showed that ouabain-sensitive Li^+ influx into Na^+ rich frog skeletal muscle was reduced by half when the K^+ of the medium was increased from 0 to 15 mM. Using the same preparation and the same high concentrations of Li^+ (substitution for Na^+), Beaugé systematically investigated this interaction of Li^+ with the Na-pump. The author concluded that Li^+ had a K^+ like effect on the pump. However K_m for Li^+ was much higher and $V_{\max}$ much lower than for K^+ . Li^+ was sometimes found to increase the stimulation of K^+ on the pump, but this effect could not be quantitated in any consistent way (Beaugé, 1975).

Several authors including Skou (1965), have studied the effects of Li^+ on preparations of Na-K-ATPase from nervous tissue (e.g. Ploeger, 1974a; Tobin et al., 1974; Robinson, 1975; Hesketh et al., 1977). Ploeger (1974a) used homogenates of cervical vagi from rats; there was no further stimulation of the enzyme by 10 mM Li^+ when the Na^+ and K^+ concentrations were optimal for enzyme activity, whereas 10 mM Li^+ caused a weak stimulation when the K^+ of the assay was reduced from

10 mM to 1 mM. Tobin et al. (1974, Fig. 2) got almost the same results using a Na-K-ATPase from rat brain. Robinson (1975) could account quantitatively for the results of a careful kinetic analysis on the effects of Li^+ , K^+ and Na^+ on the same preparation by assuming 1) a displacement by Li^+ of K^+ from α -sites where K^+ inhibits and where Li^+ may itself exert a weak stimulation; 2) an effect of Li^+ on β -sites for which K^+ has a high affinity and which would not be stimulated by Li^+ in the presence of optimal concentrations of K^+ and Na^+ ; 3) a displacement of Na^+ from its low affinity discharge site. The sites involved in the effects of Li^+ would all be on the outside of the intact cell membrane, corresponding to results by most earlier investigators. Hesketh et al. (1977, Table 5) found no effect of Li^+ in vitro on ATPase activity in various subcellular fractions from rat brain. However, the concentrations of Li^+ used were much lower than in the previous experiments.

2) Ion exchange between K^+ and Li^+ is unlikely as an explanation of the lowered K^+ in the tissue observed in the presence of Li^+ in the medium in the experiments of Israel et al. (1966, Table 4) and Kjeldsen et al. (1973, Table 1) as can be seen from the quantitative relationships.

3) In the experiments mentioned earlier (Christensen, 1974) K^+ and Li^+ were stated to exhibit similar accumulation curves (l.c. p. 1300, Column 1 and Fig. 1C). However, the similarity was not defined or investigated further, and the accumulation of Na^+ and K^+ was the same in Li^+ treated rats and control rats so that these experiments do not indicate competition in the binding.

The previous review of the literature leaves no doubt that in excitable tissue, also, Li^+ may enter the cell by means of the Na-pump and may itself exert a slight stimulation of the pump (Beaugé & Ortiz, 1972; Smith, 1974; Beaugé, 1975; Haugaard et al., 1975; Bianchi et al., 1977). The present experiments (Fig. 4.2, Table 4.3) showing a high tissue concentration of

Li^+ in the absence of K^+ could be due to this mechanism. However, absence of K^+ in the medium inhibits the Na-pump, and in cerebral slices changes in the K^+ , Na^+ and swelling that were qualitatively similar to the present results have been produced by the absence of K^+ alone (Joanny & Hillman, 1964; Okamoto & Quastel, 1970), indicating that these changes are not caused solely by Li^+ . On the other hand, the K^+ of the slices is too high and the Na^+ too low to be consistent with the activity of the Na-pump being completely abolished, and significant artifacts in the measurements of these ions in the present thesis have been tested for and ruled out (Chapter 2.2); this might indicate a relatively strong stimulation by Li^+ . However, as pointed out earlier (Chapter 2.1B i)c), leakage of K^+ from the slices would probably render the present media approx. 1 mM in K^+ . Beaugé (1975) carefully controlled for this effect which accounted for approx. half the stimulation in his preparation. Thus the effect observed in the 'absence' of K^+ is likely to express a combined effect of Li^+ and K^+ , also described by Bianchi *et al.* Compared to most similar experiments in the literature, the media of the present experiments had a relatively high volume; thus the effect of K^+ leakage must have been at least as great in the other experiments above in which it was not controlled. The effect of ouabain in lowering the Li^+ of the slices is consistent with the suggested uptake of Li^+ by the Na-pump. However, ouabain also has an effect on the metabolism of cerebral slices (Bourke & Tower, 1966b) and the effect of ouabain in the present experiments was not different from that of anoxia, except for causing an even more pronounced swelling. Therefore, the effect might as well be one of reducing the electrochemical potential of the cells and affecting the passive distribution of Li^+ .

The non-linear relationship between the K^+ of the medium and the Li^+ , Na^+ and K^+ of the slices is suggestive of a carrier mediated transport of all these ions, possibly the Na-pump,

whereas ion exchange between Li^+ and K^+ cannot explain the changes of concentrations (Table 4.3).

Sodium ions: 1) It has been a consistent finding that stimulation by Li^+ of the Na-site of the Na-pump is very ineffective in excitable tissue (e.g. Baker, 1965; Gardner & Kerkut, 1968; Thomas, 1969). Ploeger (1974 b), estimating the Na^+ content and - like Thomas (1969) - the activity of the electrogenic Na-pump by means of the hyperpolarization of the membrane, demonstrated competition between Li^+ and Na^+ (1.c. Fig. 1). The author has reconciled this surprising finding with those above and his own findings on Li-K competition, mentioned earlier, by assuming that Li^+ binds with the carrier at the K^+ site, diminishing the availability of the carriers for the outward Na^+ transport (Hertog & Ploeger, 1973, Fig. 5).

2) With the exception of a ouabain insensitive exchange of Li^+ with internal Na^+ in smooth muscle (Brading, 1975; Widdicombe, 1977), there seems to have been no observations in excitable tissue of the Na-Li countertransport demonstrated in red cells (Haas et al., 1975; Duhm et al., 1976).

3) In excitable cells, specific channels for Na^+ , K^+ and Ca^{2+} are currently held to be the device by means of which the extremely rapid passage of these ions takes place during the action potential. (Christensen, 1975, p. 200 - 203; Hille, 1976). In various preparations it has been known for a long period that Li^+ is the only cation that can replace Na^+ in the medium and maintain excitability for a limited period. The experiments by Gardner & Kerkut (1968) on snail neuron, besides demonstrating the inability of Li^+ to stimulate the Na^+ site, also showed that Li^+ could carry the inward current during the action potential. In cultured mouse neuroblastoma cells, Richelson (1977) demonstrated that the uptake of low concentrations of Li^+ was stimulated by the alkaloid veratridine and blocked by tetrodotoxine, both of which are

used to characterize the Na^+ channel. Interestingly, the concentration ratio of Li^+ between the cells and the medium in the presence of veratridine was near 10 (l.c. Fig.1), which is approx. the ratio predicted by the Nernst equation for a resting potential about 60 mV if Li^+ were distributed passively.

In the present experiments (Fig. 4.3), the linear relationship and the high regression coefficient suggest that there is little or no extrusion of the extra Na^+ added. The deviating value at 160 mM makes the regression somewhat lower than would be expected from the water content of the slices. A similar passive treatment of Na^+ by cerebral slices was observed by Hillman et al. (1974). The Li^+ of the slices did not change significantly, and the apparent lowering of the Li^+ from 120 mM to 140 mM Na^+ in the medium is far from the one-to-one relationship that would be expected for a Li^+ - Na^+ countertransport. Extension of the range of Na^+ concentrations in the media to 78 mM to 250 mM did not result in any interaction between Li^+ and Na^+ (Kjeldsen et al., 1973).

Calcium ions: The measurements of Ca^{2+} in cerebral slices by Lolley (1963) and Tower (1968) are in general agreement and likely to be applicable to the present experiments also. In the 'absence' (approx. 0.2 mM) of Ca^{2+} , tissue levels of Ca^{2+} were approx. 1 mM; otherwise, the Ca^{2+} concentrations of the slices were 2 - 3 times those of the media. The amount of free intracellular Ca^{2+} as estimated by means of the aequorin reaction, is extremely low (approx. 0.3 μM) in squid axon (Baker & Reuter, 1975 p. 14) and probably in cerebral slices also.

A review of the literature indicates three different ways, in which Li^+ may interact with Ca^{2+} . Besides that, indirect effects by means of the Na-pump or on the metabolism of slices will be discussed, as they might also explain the results.

- 1) The existence of a Ca^{2+} activated ATPase and its coupling

with Ca^{2+} transport has been demonstrated in various types of tissue (Baker & Reuter, 1975, p. 20 - 23; Christensen, 1975, p. 287 - 289). Little is known about the effects of Li^+ on this system in excitable tissue. De Meis (1971) studied the Ca^{2+} uptake in a microsomal preparation from skeletal muscle. At low concentrations of ATP or Ca^{2+} , when Ca^{2+} transport and ATPase activity are uncoupled, Li^+ inhibited the uptake more than did Na^+ or K^+ and seemed to do so primarily by preventing the binding of Ca^{2+} to the membrane. This mechanism is unlikely to explain the present effects of low concentrations of Li^+ . First, the necessary concentrations of Li^+ in the experiments by de Meis were high (substitution of Na^+), secondly, omission of Ca^{2+} in the medium did not affect the concentrations of high energy phosphates in cerebral slices (Stahl & Swanson, 1972).

2) Ca-Na ion countertransport in squid axon and other excitable tissue seems to be the most important regulator of intracellular Ca^{2+} . The Ca^{2+} efflux is reduced by replacing external Na^+ by Li^+ , but also by choline, so that the effect is rather one of absence of Na^+ . The Ca^{2+} influx associated with Na^+ efflux is increased by the presence of Li^+ , but also by K^+ and Cs^+ , again demonstrating that this effect is not specific for Li^+ (Baker & Reuter, 1975, p. 23 - 33, Fig. 10). Partridge & Thomas (1974) found an increased K^+ conductance, decreasing the membrane excitability and stabilizing the cell, after injection of Li^+ into snail neurons. They speculated that the mechanism might be one of increase in the intracellular ionized Ca^{2+} because of partial substitution of Li^+ for Na^+ in the Ca-Na countertransport. However, the actual intracellular Li^+ concentrations were shown later to be lower than the assumed concentrations of 1-12 mM, and therefore unlikely to cause significant reduction of intracellular Na^+ (Thomas et al., 1975). As the Ca^{2+} efflux is reduced by Li^+ (Baker & Reuter, 1975) a Ca-Li ion exchange is also unlikely to explain the present results.

3) Kluge et al. (1974) observed binding of Li^+ to a preparation of cerebral microsomes; the binding was strongly influenced by Ca^{2+} in concentrations of 0.25 mM to 1 mM. Correspondingly, 1.5 mM Li^+ in the medium caused a 10 - 25% reduction in the binding of Ca^{2+} to the microsomal preparation. The binding curves of Li^+ exhibited S-shape (l.c. Fig. 2 & 5) not noticed in the former or commented on in the latter by the authors, but suggesting conformational changes of the binding protein. The authors speculated that the clinical effect of Li^+ might be due to a reduction of membrane-bound Ca^{2+} . However, at saturation, the subcellular preparation used by the authors bound approx. ten times the concentration of Ca^{2+} found in vivo in that fraction, implying that one should exercise strong reservations about such extrapolations to the situation in vivo. Also, the binding of Li^+ to the preparation was not very strong, as two washings with distilled water abolished the binding completely.

In the present experiments low concentrations of Ca^{2+} or K^+ had somewhat similar effects on the concentrations of Li^+ , K^+ and Na^+ , and on the swelling of cerebral slices (Fig. 4.4 and 4.2). However, by means of the regression analysis (see Methods for criteria) a statistical distinction could be made between the Li^+ curves, the first being linear and the latter being non-linear; though empirical, such a distinction might well indicate different mechanisms of uptake. The interaction between Li^+ and K^+ was found likely to be due, at least partly, to the Na-pump. An inhibition of the Na-pump cannot explain the results of Fig. 4.4, because Ca^{2+} itself is an inhibitor of this mechanism (e.g. Skou, 1965, p. 598; Christensen, 1975, Fig. 9.6). Li^+ itself cannot have caused the changes either, because similar changes of K^+ and Na^+ of cerebral slices and isolated retinas were seen in Ca^{2+} deficient media (Joanny & Hillman, 1964; Ames et al., 1967).

Absence of Ca^{2+} from the medium has been shown to stimulate the O_2 -uptake and glycolysis of cerebral slices (Dickens &

Greville, 1935; Krebs, 1950; Bourke & Tower, 1966b). As was shown earlier (Chapter 2.1B ii) & v)) omission of glucose from the medium reduced the high Li^+ in the slices seen in the absence of Ca^{2+} . This might indicate that the effect on Li^+ is due to increased metabolism. However, high concentrations of K^+ , which are also known to stimulate respiration of cerebral slices (Dickens & Greville, 1935; Hertz & Schou, 1962) decreased the Li^+ of the slices in the present experiments (Fig. 4.2, $\text{K}^+ = 10 \text{ mM}$) and in those of Kjeldsen et al. (1973, p. 112, para. 4), who used concentrations up to 85 mM. Furthermore, as was mentioned previously, dependence of the Li^+ concentrations on metabolism may as well be due to dependence on the electrochemical potential of the cells. The Ca^{2+} concentration of the medium can be varied widely, without affecting the resulting potential (Hillman & McIlwain, 1961). Therefore, lowering of the Ca^{2+} of the medium might cause leakage or passage via channels of Li^+ into cells with intact resting potentials, as was observed by Richelson (1977). Finally, a binding to subcellular structures, as observed by Kluge et al. (1974), might explain the results.

Magnesium ions: Within the concentration ranges tested, the effects of Mg^{2+} in the medium on the Li^+ and K^+ in the slices were opposite, although not equal to those of K^+ and Ca^{2+} in the medium. There seems to be no previous publications of any reasonable relevance on interaction of transport between Li^+ and Mg^{2+} in vitro, although there is some evidence of interaction of Li^+ and Mg^{2+} in vivo and much evidence of interaction in Mg-dependent enzymatic reactions. (For reviews, see Hullin, 1975, p. 369 - 370, and Birch, 1978, p. 97 - 101).

Preliminary conclusions: In the previous discussion, the mechanisms known to operate in the transport of K^+ , Na^+ and Ca^{2+} have been reviewed and evidence from the literature of their involvement in the uptake of Li^+ has been presented. The present results with K^+ are compatible with some competition between Li^+ and K^+ about (one of) the K^+ -site(s) of the

Na-pump. Major interactions between Li^+ and Na^+ do not take place in cerebral slices. An interaction between Li^+ and Ca^{2+} was clearly observed in the present experiments, but its nature is uncertain, although some known mechanisms of transport can be ruled out. The importance of Ca^{2+} for membrane permeability (Baker & Reuter, 1975, p.9) suggests leak diffusion as an explanation, but competition between Li^+ and Ca^{2+} in the binding to subcellular structures has also been observed in other preparations. The magnesium experiments showed an interaction opposite to that of Ca^{2+} . Collectively, the results cannot be explained solely as either competition between Li^+ and K^+ about a single carrier, or as being due to leakage because of defective extrusion of Li^+ . Clearly, kinetic experiments using a broader range of Li^+ concentrations would have to be performed in order to characterize the interactions observed.

SUMMARY

1. The concentration of Li^+ in the tissue was linearly related to that of the medium (0.5 - 1.5 mM Li^+) with a concentration ratio of approx. 1.5. In the absence of glucose or oxygen the ratio approached unity.
2. The concentration ratio of Li^+ increased non-linearly as the concentration of K^+ in the medium was decreased within a range of 0 - 10 mM K^+ . In the absence of K^+ in the medium, the concentration ratio of Li^+ was 50% higher than in the presence of 5 mM K^+ . These findings are compatible with competition of Li^+ and K^+ , about a K^+ site of the Na-pump.
3. There was an inverse linear relationship between the concentration of Li^+ in the slices and that of Ca^{2+} in the medium within a range of 0 - 5 mM. None of the known transport mechanisms for Ca^{2+} can account for these results.
4. The concentration of Li^+ in the slices increased by 10% when the Mg^{2+} of the medium was increased from 1.3 mM to 2.6 mM.

5. Changes of the concentration of Na^+ between 120 mM and 170 mM in the medium had no significant effect on the Li^+ of the slices.

6. In agreement with earlier reports, the K^+ of the slices was lower in the standard media containing Li^+ 1.5 mM than in the absence of Li^+ , but the difference did not reach statistical significance.

A STUDY OF THE INITIAL UPTAKE OF HIGH CONCENTRATIONS OF Li^+
INTO CEREBRAL SLICES AND ITS DEPENDENCE ON K^+ AND Ca^{2+}

In the experiments of Chapter Three, saturation of the uptake of Li^+ in cerebral slices was not seen in the presence of 0.5 mM to 2 mM Li^+ in the medium. However, this might be because the concentrations were too low. The experiments of Chapter Four clearly demonstrated interactions of Li^+ with K^+ and Ca^{2+} at steady state. However, the interactions, which were interpreted as different mechanisms of uptake, could not be distinguished except by the absence and presence of a linear relationship of Li^+ in the slices with K^+ and Ca^{2+} in the media, respectively.

In the following experiments concentrations of Li^+ up to 120 mM were used. The aim of these experiments was to investigate the possibility of a saturable uptake of Li^+ and to characterize kinetically the interactions between Li^+ and K^+ , and Li^+ and Ca^{2+} , i.e. the possibility of demonstrating one or two carriers used in the uptake of Li^+ , and, if this could be demonstrated, to characterize the inhibition exerted by K^+ and Ca^{2+} towards Li^+ .

METHODS

Preparation of tissue and incubation: Cerebral slices were prepared as described earlier, twelve in each experiment.

In the experiments using Li^+ 1 mM to 30 mM (medium-high concentrations) the slices were pre-incubated in 10 ml of either a standard medium (Table 2.2) or in media with different concentrations of K^+ or Ca^{2+} (Table 4.3 and 4.4). Li^+ solu-

tions were then added as described in Chapter Three. Neither the volumes added which were maximally 2 - 3 %, nor the different osmolarity caused by the additions were considered important.

In the experiments correcting for passive uptake, in which concentrations of Li^+ of 40 mM, 64 mM, 96 mM and 120 mM (high concentrations) were used, the solutions were prepared in advance by adjusting the concentration of NaCl in such a way that the total osmolarity of Li^+ containing and Li^+ free media was the same (approx. 380 mosm.). This high osmolarity was accepted in order to avoid very low concentrations of Na^+ in the Li^+ -media. As the LiCl stock solutions were made by adding HCl to Li_2CO_3 , they were acid. This acidity became significant when large quantities of the solutions were included in the media; therefore, the LiCl solutions were neutralized by the addition of solid NaOH.

The six incubation periods for the initial uptake of the medium-high concentrations (min) were: 1.0; 1.5; 2.0; 2.5; 3.0 and 4.0. The incubations using high concentrations of Li^+ were carried out by transferring the slices from the Li^+ free media to Li^+ containing media for 2.33 min, this being the mean value of the incubation times mentioned above.

Measurement of Li^+ : The concentrations of Li^+ in the cerebral slices and the media were measured as described earlier, except that the volumes of TCA were higher. For unknown reasons, the concentrations of Li^+ in the media sometimes differed somewhat from those intended, whereas the precision of the measurements was the same as usual (S.D. % less than 2%). Several calculations depended critically on these concentrations. Therefore, in contrast to earlier experiments, the measured concentrations were used in the calculations instead of the nominal ones (Table 5.1 - 5.8, column (i)).

Calculations: In order to test the possibility of saturable

diffusion, the conventional double reciprocal regression of the Michaelis-Menten equation was used (Lineweaver & Burk, 1934), and statistical weights applied as shown in Appendix I. The unidirectional influx at each concentration of Li^+ and Ca^{2+} , or Li^+ and K^+ , was estimated by fitting straight lines to the cerebral concentrations of Li^+ measured during the initial uptake, as described in Chapter Three. Only regression lines that were significantly different from null by a P-value of less than 5% were included. Apart from replacement of estimates of the influx thus discarded, each estimate was based on a single experiment (six slices).

RESULTS

The use of the Lineweaver-Burk regression: The estimates of the influx of Li^+ under various experimental conditions is shown (Table 5.1 - 5.8, column (ii)). This was measured in the presence of 8 - 10 different concentrations of Li^+ from 1 mM to approx. 30 mM. At each of these concentrations of Li^+ , the K^+ concentrations of the media were varied from 0 mM to 10 mM (Table 5.1 - 5.4) and the Ca^{2+} concentrations from 0 mM to 5.2 mM (Table 5.5 - 5.8). Evaluation of the kinetics by means of the Lineweaver-Burk regression resulted in small numerical differences between the slopes and between the intercepts (Table 5.1 - 5.8, section II). There were no significant differences associated with changes of K^+ (Table 5.1 - 5.4). When Ca^{2+} in the medium was varied, the slopes increased with its concentration, but only the slope measured in the presence of Ca^{2+} 10 mM (Table 5.8 II) was significantly different from the other values; two of the intercepts were significantly different from zero (Table 5.5 II and 5.7 II). Comparison of the two control values (Table 5.3 II and 5.7 II) suggests reservations about these significances that are due to the small standard deviations resulting from the weighting procedure. An alternative method (see Appendix I) was applied to the results of Table 5.1 (section II, values

in brackets). This resulted in a very different estimate of the slope and intercept and indicates a poor fit of the L-B model used (see Discussion). In view of these uncertainties it would have no meaning to calculate the estimates of K_m and V_{max} .

Correction for passive uptake: The influx values above have been treated as if the membrane limited uptake was either saturable or non-saturable. However, the uptake might well be both saturable and non-saturable (Christensen, 1975, p. 133 - 141), and this might be the reason for the conflicting results of the last section. Consequently, an attempt was made to correct the influx for passive uptake by means of eq(7) in Appendix II.

The values of the corrected influx are shown (Table 5.1 - 5.8, column (v)). At the highest and lowest concentrations of K^+ (Table 5.1 and 5.4) and Ca^{2+} (Table 5.5 and 5.8) there were one to several negative values, suggesting no difference from passive uptake. In the remaining cases the Lineweaver-Burk regressions were estimated. The intercept of one of the control values (Table 5.7, section IV) was significantly different from zero, but as none of the others were (including the other control, Table 5.3, section IV) this is unlikely to indicate saturation. Consequently, K_m and V_{max} were not calculated.

Sources of error: Two sources of systematic error in the correction for passive uptake were primarily considered.

First, the values of n in the presence of 120 mM Li^+ (Table 5.9(ii)) differed from the corresponding values of $n' = 1.3-1.7$ determined at low concentrations of Li^+ in a standard medium; in the absence of Ca^{2+} or K^+ , the difference between n and n' was even greater (see Chapter Four and Appendix II, Notations). In experiments using standard media n' -values were found to decrease gradually with increase of concentration in the presence of Li^+ above 5 mM, and to reach

the value of n asymptotically (not shown). It might then not be justified to use the same value of n both in the calculation of d and in the correction of the influx at concentrations between 1 mM and 30 mM. From eq (7), Appendix II: $v = (Bt/n - dC_{\text{med}}) c$, it can be seen that higher values of n in the correction of Bt would result in reduced values of v if d remained unaltered. Therefore, in order to assess whether the dependence of n' on the Li^+ concentration could explain the remaining positive values of v , it was calculated from eq (7) how high values of n' would be required in order to obtain values of v equal to or less than zero (Table 5.2, 5.3, 5.6 and 5.7 column (vi)). Owing to experimental variation they are not uniform within the tables; however, it is clear that the low values of n' are represented in Table 5.2 and 5.6, whereas the values of n' in the controls would not have been observed in actual measurements.

Secondly, the uptake of Li^+ might be altered by the use of high concentrations of Li^+ and lower concentrations of Na^+ than those examined in Chapter Four (Fig. 4.3) or by the high total osmolality of the solutions. Therefore, concentrations of Na^+ of 60 - 180 mM were used and slices were incubated for 2.33 min in the presence of Li^+ 5 mM and approx. 50 mM. The swelling of the slices was more pronounced in the media of low osmolality, whereas high osmolality due to Li^+ or Na^+ had no influence on the swelling, neither in these experiments nor in any other experiments of the present chapter. Neither the concentrations of Na^+ , nor the total osmolality had any influence on the relative uptake of Li^+ , nor was the relative uptake of 5 mM Li^+ significantly different from that of 50 mM (Table 5.10).

Calculation of transfer constants: As the experiments did not indicate saturable uptake or a combination of saturable and non-saturable uptake, the uptake was considered independent of the concentration of Li^+ in the medium. A mean trans-

fer constant was calculated for each concentration of K^+ and Ca^{2+} as $\text{Sum}(B/C_{\text{med}})/N$, in which B and C_{med} are corresponding values in Table 5.1 - 5.8 column (ii) and (i), respectively, and N is the number of experiments in one table. The results are shown (Table 5.1 - 5.8, section V). Comparison of the transfer constants from the eight tables by the analysis of variance demonstrated no significant differences between them. Consequently they were considered estimates of the same value $(9.42 \pm 1.04)10^{-2} \text{ min}^{-1}$ (mean $\pm$ S.D. of eight estimates). The mean transfer constant obtained from the estimates of Table 3.1 (v) was $(8.75 \pm 0.65) 10^{-2} \text{ min}^{-1}$ (mean $\pm$ S.D. of six estimates), which is not significantly different from the mean transfer constant of this chapter.

Tables 5.1 - 5.8: The Li^+ uptake was measured at various intervals from 1 - 4 min (see Methods). Concentrations of Li^+ of approx. 1 mM to 30 mM were used, as shown in the tables. At each of these concentrations of Li^+ the K^+ of the medium was varied from 0 mM to 10 mM (Tables 5.1 - 5.4) and the Ca^{2+} concentration from 0 mM to 5.2 mM (Tables 5.5 - 5.8).

I. The values of (i) are based on the number of samples shown in brackets. Those of (ii-iv) are based on 6 slices, whose Li^+ concentrations were used in the regression analysis leading to the values. From the variance of the slopes (iii) and the slopes themselves (ii) a statistical weight, $w = B^4/s^2(B)$ can be calculated, which was used in the Lineweaver-Burk regression, as shown in Appendix I. The values necessary for the calculation of (v), besides B and A in (ii) and (iv), are shown in Table 5.9 and the calculation is shown in Appendix II. The theoretical values of n' shown in an additional column (vi) in four tables are explained in the text (see Results).

II. The results of the Lineweaver-Burk plot were obtained by regression analysis using statistical weights (w) as described in Appendix I.

III. The combined intercept, $\bar{a}$, which expresses approx. the percentage of Li^+ in the extracellular space and adhering fluid of the slices, was obtained from the corresponding values of column (iv) and (i) and $\text{Sum}(A/C_{\text{med}})/N$, in which N is the number of experiments in each table.

IV. The Lineweaver-Burk regressions were worked out only if all the values of (v) were positive. The results were obtained as described in II.

V. The transfer constant was obtained from the corresponding values of column (ii) and (i) as $\text{Sum}(B/C_{\text{med}})/N$, in which N is the number of experiments in each table.

TABLE 5.1 INITIAL UPTAKE OF Li^+ , K^+ IN THE MEDIUM = 0 mM

I	(i) Li^+ in media, C_{med} (mmol/l)	(ii) Influx of Li^+ , B (mmol/(kg min))	(iii) Variance of influx, s^2 (B)	(iv) Intercept of influx, A (mmol/kg)	(v) Corrected influx, v (mmol/(kg min))
	1.042 (4)	0.0983	0.93×10^{-3}	0.2207	0.0040
	1.538 (8)	0.1429	1.3×10^{-3}	0.2664	0.0035
	2.029 (8)	0.2787	0.84×10^{-3}	0.2705	0.1057
	2.857 (8)	0.2728	2.5×10^{-3}	0.5599	0.0147
	4.996 (4)	0.3261	0.012	1.081	-0.1438
	6.161 (4)	0.5307	0.015	1.406	-0.0331
	10.22 (12)	0.7391	0.019	1.555	-0.2133
	16.15 (8)	2.010	0.13	3.887	0.6076
	21.67 (8)	2.657	0.35	2.551	0.7705
	27.06 (24)	4.166	0.22	1.561	1.913
II	Lineweaver-Burk plot of values from (ii): $X^2 = 2.107$; $Y^2 = 175.3$; $XY = 18.44$; $\bar{x} = 0.04323$; $\bar{y} = 0.3092$; $S_w = 1669$; $B' = 8.75 \text{ kg min/l} + 0.69$; $A' = -0.07 \text{ kg min/mmol} \pm 0.04$. (Alternative method, see Appendix I: $B' = 15.78 \text{ kg min/l} + 1.56$; $A = -0.3456 \text{ kg min/mmol} \pm 0.068$)				
III	Combined intercept, $\bar{a}$, (mean $\pm$ S.D.): $17.27\% \pm 5.76$ ($N = 10$)				
IV	Lineweaver-Burk plot of values from (v): not calculated.				
V	Estimated transfer constant (mean $\pm$ S.D.): $(10.45 \pm 2.88) 10^{-2} \text{ min}^{-1}$ ($N = 10$)				

TABLE 5.2 INITIAL UPTAKE OF Li^+ , K^+ IN THE MEDIUM = 2.5 mM

I	(i)	(ii)	(iii)	(iv)	(v)	(vi)
Li^+ in media,	Influx	Variance	Intercept	Corrected		
C_{med}	of Li^+ , B	of influx,	of influx, A	influx, v		
(mmol/l)	(mmol/(kg min))	$s^2(B)$	(mmol/kg)	(mmol/(kg min))	n'	
1.035 (5)	0.0524	0.37×10^{-3}	0.2180	0.0147	1.4	
1.512 (11)	0.0798	0.67×10^{-3}	0.2891	0.0248	1.4	
2.051 (13)	0.2074	0.34×10^{-3}	0.2814	0.1376	2.8	
2.857 (8)	0.2607	3.1×10^{-3}	0.5193	0.1621	2.4	
4.996 (4)	0.2829	5.7×10^{-3}	1.006	0.1022	1.6	
6.161 (4)	0.7315	4.5×10^{-3}	0.6622	0.5271	3.2	
10.02 (4)	0.4922	0.019	2.501	0.1261	1.4	
16.15 (8)	2.327	0.48	3.130	1.811	3.8	
21.67 (8)	1.612	0.16	4.097	0.8466	2.0	
26.61 (6)	2.466	0.15	2.835	1.550	2.6	

II Lineweaver-Burk plot of values from (ii):

$\chi^2 = 1.953$; $Y^2 = 190.9$; $XY = 18.43$; $\bar{x} = 0.06850$; $\bar{y} = 0.6658$; $SW = 424.7$; $B' = 9.44 \text{ kg min/l} \pm 0.72$;

$A' = 0.02 \text{ kg min/mmol} \pm 0.07$.

III Combined intercept, $\bar{a}$, (mean $\pm$ S.D.): $17.69\% \pm 4.60$ ($N = 10$)IV Lineweaver-Burk plot of values from (v): $\chi^2 = 0.3531$; $Y^2 = 67.55$; $XY = 4.604$; $\bar{x} = 0.07708$;

$\bar{y} = 1.004$; $SW = 75.20$; $B'' = 13.04 \text{ kg min/l} \pm 1.68$; $A'' = -0.001 \text{ kg min/mmol} \pm 0.17$

V Estimated transfer constant (mean $\pm$ S.D.): $(8.31 \pm 2.23) 10^{-2} \text{ min}^{-1}$ ($N = 10$)

TABLE 5.3

INITIAL UPTAKE OF Li^+ , K^+ IN THE MEDIUM = 5 mM

(CONTROL VALUE)

I	(i)	(ii)	(iii)	(iv)	(v)	(vi)
Li^+ in media,	Influx		Variance	Intercept	Corrected	
C_{med}	of Li^+ , B		of influx,	of influx, A	influx, v	n'
(mmol/l)	(mmol/(kg min))	s^2 (B)	s^2 (B)	(mmol/kg)	(mmol/(kg min))	
1.042 (4)	0.0682	0.55×10^{-3}		0.2002	0.0430	2.6
1.538 (8)	0.1629	0.90×10^{-3}		0.1664	0.1277	4.0
2.029 (8)	0.1551	1.5×10^{-3}		0.3583	0.1067	3.0
2.857 (8)	0.2731	3.6×10^{-3}		0.3773	0.2068	3.6
4.996 (4)	0.3747	2.4×10^{-3}		1.010	0.2553	3.0
6.161 (4)	0.8235	0.015		0.7889	0.6883	5.2
10.22 (12)	0.8100	0.025		1.606	0.5672	3.0
16.15 (8)	2.188	0.17		2.666	1.835	5.0
21.67 (8)	2.106	0.14		2.775	1.604	3.8
27.06 (24)	2.149	0.26		4.671	1.506	3.0

II Lineweaver-Burke plot of values from (ii): $\chi^2 = 1.070$; $Y^2 = 119.3$; $XY = 10.52$; $\bar{x} = 0.06594$;

$\bar{y} = 0.6258$; $Sw = 416.2$; $B' = 9.84 \text{ kg min/l} \pm 0.97$; $A' = -0.02 \text{ kg min/mmol} \pm 0.08$

III Combined intercept $\bar{a}$ (mean $\pm$ S.D.): $15.62\% \pm 3.10$ (N = 10)

IV Lineweaver-Burk plot of values from (v): $\chi^2 = 0.3504$; $Y^2 = 61.90$; $XY = 4.105$; $\bar{x} = 0.06847$;

$\bar{y} = 0.7758$; $Sw = 146.2$; $B'' = 11.71 \text{ kg min/l} \pm 1.69$; $A'' = -0.03 \text{ kg min/mmol} \pm 0.14$

V Estimated transfer constant (mean $\pm$ S.D.): $(9.43 \pm 2.44) 10^{-2} \text{ min}^{-1}$ (N = 10)

TABLE 5.4 INITIAL UPTAKE OF Li^+ , K^+ IN THE MEDIUM = 10 mM

I	(i)	(ii)	(iii)	(iv)	(v)
Li^+ in media, C_{med}	Influx of Li^+ , B	Variance of influx, s^2 (B)	Intercept of influx, A	Corrected influx, v	
(mmol/l)	(mmol/(kg min))		(mmol/kg)	(mmol/(kg min))	
1.003 (4)	0.1111	0.37×10^{-3}	0.1695	0.0214	
1.512 (11)	0.0742	0.39×10^{-3}	0.2542	-0.0736	
2.029 (8)	0.1527	0.72×10^{-3}	0.2889	-0.0385	
2.857 (8)	0.3970	3.4×10^{-3}	0.2599	0.1523	
4.996 (4)	0.4864	1.6×10^{-3}	0.4244	0.0304	
6.161 (4)	0.8049	9.6×10^{-3}	0.2517	0.2703	
10.02 (4)	0.9832	0.053	1.549	0.0698	
16.15 (8)	1.434	0.19	3.657	-0.0586	
21.67 (8)	1.626	0.055	2.753	-0.4170	
27.06 (24)	2.269	0.16	2.987	-0.2499	

II Lineweaver-Burk plot of values from (ii): $x^2 = 2.303$; $y^2 = 177.5$; $\bar{x} = 19.24$; $\bar{y} = 0.07747$;
 $\bar{y} = 0.8054$; $S_w = 419.9$; $B' = 8.35 \text{ kg min/l} \pm 0.66$; $A' = 0.16 \text{ kg min/mmol} \pm 0.07$.

III Combined intercept, $\bar{a}$, (mean $\pm$ S.D.): 13.15 ± 5.26 (N = 10)

IV Lineweaver-Burk plot of values from (v): not calculated.

V Estimated transfer constant (mean $\pm$ S.D.): $(9.48 \pm 2.69) 10^{-2} \text{ min}^{-1}$ (N = 10)

TABLE 5.5

INITIAL UPTAKE OF Li^+ , Ca^{2+} IN THE MEDIUM = 0 mM

I	(i) Li^+ in media, C_{med} (mmol/l)	(ii) Influx of Li^+ , B (mmol/(kg min))	(iii) Variance of influx, $s^2(\text{B})$	(iv) Intercept of influx, A (mmol/kg)	(v) Corrected influx, v (mmol/(kg min))
	1.034 (4)	0.1783	0.25×10^{-3}	0.1596	0.0971
	2.124 (6)	0.2181	2.3×10^{-3}	0.2834	0.0344
	3.090 (4)	0.2178	3.1×10^{-3}	1.088	-0.0608
	4.870 (4)	0.4888	5.0×10^{-3}	1.669	0.0664
	7.149 (3)	1.047	0.047	1.207	0.4646
	9.909 (4)	0.6987	0.033	3.506	-0.1945
	16.63 (12)	1.597	0.11	4.654	0.1464
	20.46 (17)	1.935	0.34	5.043	0.1469
	28.80 (14)	2.423	0.21	5.030	-0.1283
II	Lineweaver-Burk plot of values from (ii): $X^2 = 4.027$; $Y^2 = 164.6$; $XY = 24.62$; $\bar{x} = 0.07164$; $\bar{y} = 0.6828$; $Sw = 314.5$; $B' = 6.12 \text{ kg min/l} \pm 0.50$; $A' = 0.24 \text{ kg min/mmol} \pm 0.07$				
III	Combined intercept, $\bar{a}$, (mean $\pm$ S.D.): 24.86 ± 8.80 (N = 9)				
IV	Lineweaver-Burk plot of values from (v): not calculated.				
V	Estimated transfer constant (mean $\pm$ S.D.): $(10.42 \pm 3.41) 10^{-2} \text{ min}^{-1}$ (N = 9)				

TABLE 5.6 INITIAL UPTAKE OF Li^+ , Ca^{2+} IN THE MEDIUM = 1.3 mM

I	INITIAL UPTAKE OF Li^+ , Ca^{2+} IN THE MEDIUM = 1.3 mM					
	(i) Li^+ in media, C_{med} (mmol/l)	(ii) Influx of Li^+ , B (mmol/(kg min))	(iii) Variance of influx, s^2 (B)	(iv) Intercept of influx, A (mmol/kg)	(v) Corrected influx, v (mmol/(kg min))	(vi) n'
II	1.034 (4)	0.0948	0.65×10^{-3}	0.1858	0.0361	1.6
	2.006 (4)	0.1405	2.5×10^{-3}	0.5616	0.0231	1.2
	3.090 (4)	0.3023	4.1×10^{-3}	0.5344	0.1283	1.6
	4.870 (4)	0.6191	7.8×10^{-3}	0.8497	0.3565	2.2
	7.149 (3)	1.055	0.068	0.5818	0.6812	2.4
	9.909 (4)	1.070	0.063	2.037	0.5203	1.8
	16.63 (12)	1.950	0.16	2.561	1.040	2.0
	18.80 (9)	1.836	0.24	4.154	0.7773	1.6
	28.80 (14)	2.721	0.13	4.251	1.092	1.6
	Lineweaver-Burk plot of values from (ii): $X^2 = 1.051$; $Y^2 = 73.89$; $XY = 8.512$; $\bar{X} = 0.05160$; $\bar{Y} = 0.4897$; $S_w = 619.6$; $B' = 8.10 \text{ kg min/l} \pm 0.98$; $A' = 0.07 \text{ kg min/mmol} \pm 0.06$.					
III	Combined intercept, $\bar{a}$, (mean $\pm$ S.D.): $17.96\% \pm 5.46$ (N = 9)					
IV	Lineweaver-Burk plot of values from (v): $X^2 = 0.06464$; $Y^2 = 10.60$; $XY = 0.7132$; $\bar{X} = 0.07278$; $\bar{Y} = 1.231$; $S_w = 22.49$; $B'' = 11.03 \text{ kg min/l} \pm 3.93$; $A'' = 0.43 \text{ kg min/mmol} \pm 0.36$.					
V	Estimated transfer constant (mean $\pm$ S.D.): $(10.57 \pm 2.26) 10^{-2} \text{ min}^{-1}$ (N = 9)					

TABLE 5.7 INITIAL UPTAKE OF Li^+ , Ca^{2+} IN THE MEDIUM 2.6 mM

I	(CONTROL VALUE)					
	(i)	(ii)	(iii)	(iv)	(v)	(vi)
Li^+ in media, C_{med} (mmol/l)	Influx of Li^+ , B (mmol/(kg min))	Variance of influx, s^2 (B)	Intercept of influx, A (mmol/kg)	Corrected influx, v (mmol/(kg min))		n'
1.034 (4)	0.1389	0.86×10^{-3}	0.1314	0.1162		5.2
2.006 (4)	0.2729	2.5×10^{-3}	0.3407	0.2290		5.2
3.101 (6)	0.1540	2.1×10^{-3}	0.4750	0.0773		2.0
4.870 (4)	0.3936	1.9×10^{-3}	1.084	0.2781		3.2
7.149 (3)	0.6248	0.027	1.385	0.4569		3.4
9.909 (4)	0.9163	0.040	1.852	0.6852		3.6
21.98 (10)	1.384	0.041	3.743	0.8498		2.4
29.24 (6)	1.742	0.273	6.695	1.028		2.4

II Lineweaver-Burk plot of values from (ii): $X^2 = 1.143$; $Y^2 = 85.51$; $XY = 9.374$; $\bar{x} = 0.07404$;
 $\bar{y} = 0.9712$; $Sw = 162.0$; $B' = 8.20 \text{ kg min/l} \pm 0.94$; $A' = 0.36 \text{ kg min/mmol} \pm 0.10$.

III Combined intercept, $\bar{a}$, (mean $\pm$ S.D.): $18.16\% \pm 3.41$ ($N = 8$)

IV Lineweaver-Burk plot of values from (v): $X^2 = 0.4021$; $Y^2 = 35.47$; $XY = 3.475$; $\bar{x} = 0.1023$;
 $\bar{y} = 1.714$; $Sw = 26.70$; $B'' = 8.64 \text{ kg min/l} \pm 1.57$; $A'' = 0.83 \text{ kg min/mmol} \pm 0.25$

V Estimated transfer constant (mean $\pm$ S.D.): $(8.79 \pm 3.26) 10^{-2} \text{ min}^{-1}$ ($N = 8$)

TABLE 5.8 INITIAL UPTAKE OF Li^+ , Ca^{2+} IN THE MEDIUM = 5.2 mM

I	(i) Li^+ in media, C_{med} (mmol/l)	(ii) Influx of Li^+ , B (mmol/(kg min))	(iii) Variance of influx, $s^2(B)$	(iv) Intercept of influx, A (mmol/kg)	(v) Corrected influx, v (mmol/(kg min))
	1.077 (2)	0.0742	0.19×10^{-3}	0.1822	0.0039
	2.006 (4)	0.1596	2.1×10^{-3}	0.5116	0.0306
	3.090 (4)	0.1712	1.9×10^{-3}	0.6295	-0.0343
	4.870 (4)	0.3478	0.78×10^{-3}	1.203	0.0309
	7.149 (3)	0.5816	0.013	1.224	0.1229
	11.71 (2)	1.273	0.068	1.103	0.5505
	16.63 (12)	1.566	0.066	2.239	0.5181
	18.80 (9)	1.379	0.083	3.844	0.1591
	29.24 (6)	2.173	0.34	4.631	0.2782
II	Lineaver-Burk plot of values from (ii): $X^2 = 0.7043$; $Y^2 = 144.2$; $XY = 9.854$; $\bar{x} = 0.07057$;				
	$\bar{y} = 0.8456$; $B' = 13.99 \text{ kg min/l} + 1.19$; $A' = -0.14 \text{ kg min/mmol} + 0.10$; $Sw = 267.4$				
III	Combined intercept, $\bar{a}$, (mean $\pm$ S.D.): $18.20\% + 5.16$ (N = 9)				
IV	Lineaver-Burk plot of values from (v): not calculated.				
V	Estimated transfer constant (mean $\pm$ S.D.): $(7.86 + 1.54)10^{-2} \text{ min}^{-1}$ (N = 9)				

TABLE 5.9 EVALUATION OF THE RATE CONSTANTS FOR THE NON-SATURABLE INFLUX (HIGH CONCENTRATIONS)

Cation concn.	(i) $n(d+\bar{a}/n) \times 10^2$, p	(ii) C_{cs}/C_{med} at steady state, n	(iii) $1-e^{-k_D t/n}$ $\times 10^2$, d	(iv) k_D $(10^{-2} \text{ min}^{-1})$	(v) Correctn. factor for calcn. of $s^2(v)$
(mM)	(%/2.33 min)	(%)	(%/2.33 min)		
$K^+ = 0$	38.49 ± 7.9 (10)	101.8 ± 2.5 (6)	20.84	10.20	1.258
$K^+ = 2.5$	26.35 ± 9.1 (8)	96.68 ± 1.0 (6)	8.957	3.89	1.098
$K^+ = 10$	34.61 ± 6.2 (10)	94.14 ± 3.6 (6)	22.80	10.44	1.288
$Ca^{2+} = 0$	45.42 ± 10.9 (10)	103.7 ± 2.6 (6)	19.83	9.82	1.242
$Ca^{2+} = 1.3$	31.82 ± 8.3 (8)	95.72 ± 2.0 (6)	14.48	6.42	1.167
$Ca^{2+} = 5.2$	33.50 ± 6.7 (10)	94.41 ± 3.0 (6)	16.21	7.15	1.190
$K^+ = 5$					
$Ca^{2+} = 2.6$	22.71 ± 8.4 (53)	96.01 ± 3.4 (6)	6.209	2.64	1.064

Cerebral slices were incubated for 2.33 min in media containing 40 mM to 120 mM Li^+ (see Methods). An experimental value (column (i)) containing the non-saturable, membrane limited, uptake, d, was found from the linear relation: $C_{cs} = (d + \bar{a}/n)n C_{med} + u$ (eq (6) Appendix II, modified). In this equation $C_{cs} = Li^+$ in the slices after 2.33 min (mmol/kg), $C_{med} = Li^+$ in the medium (mmol/l). The values of $n = C_{cs}/C_{med}$ after 60 min incubation in the presence of 120 mM Li^+ were determined in separate experiments and are shown in (ii). In both columns, the results presented are the means $\pm$ S.D. of the number of slices in brackets. The remaining expression, $\bar{a}$, necessary for calculating d was obtained from Table 5.1 - 5.8, section III. The calculated values of d at 2.3 min are shown in (iii). Finally the values of k_D were found from the expression $d = 1 - e^{-k_D t/n}$ column (iv)). The results (i-iv) were used to calculate the corrected influx of Li^+ in cerebral slices (eq. (7) Appendix II). The values in col. (v) were used as a multiplication factor to correct the variance of the influx (eq. (8) - (10) Appendix II).

TABLE 5.10 THE INFLUENCE OF Na^+ AND TOTAL OSMOLARITY ON THE INITIAL UPTAKE OF Li^+

	(i) $\text{Li}^+ = 5 \text{ mM}$ $\text{Na}^+ = 60 \text{ mM}$	(ii) $\text{Li}^+ = 5 \text{ mM}$ $\text{Na}^+ = 150 \text{ mM}$	(iii) $\text{Li}^+ = 5 \text{ mM}$ $\text{Na}^+ = 180 \text{ mM}$	(iv) $\text{Li}^+ = 48 \text{ mM}$ $\text{Na}^+ = 100 \text{ mM}$	(v) $\text{Li}^+ = 48 \text{ mM}$ $\text{Na}^+ = 150 \text{ mM}$
$C_{\text{CS}}/C_{\text{med}}$	30.3	34.7	32.5	37.6	35.5
(% per 2.33 min)	$\pm 4.0(6)$	$\pm 4.0(6)$	$\pm 3.7(5)$	$\pm 3.9(6)$	$\pm 7.1(6)$
Swelling	47.3	14.9	16.2	12.3	14.7
(% of fresh wt)	$\pm 3.8(6)$	$\pm 1.5(6)$	$\pm 2.0(5)$	$\pm 3.2(6)$	$\pm 2.1(6)$

Cerebral slices were pre-incubated for 30 min without Li^+ in a medium of standard composition (Table 2.2) except that the concentrations of Na^+ were different; Li^+ was then added as a concentrated solution (i-iii). In (iv) the slices were pre-incubated in a standard medium; in (v) the slices were pre-incubated in a Li^+ free solution like that of (iii); the slices were then transferred to media containing Li^+ and Na^+ as shown (iv-v). The incubation time in the presence of Li^+ was 2.33 min. The analysis of variance showed no significant difference between groups with respect to the relative uptake of Li^+ , whereas the swelling in the presence of low Na^+ (i) was higher than that of the other groups.

DISCUSSION

Incubation times: The initial uptake was measured between 1 min and 4 min. These times were chosen in order to avoid major systematic errors due to interference from the extracellular diffusion and back diffusion, as discussed in Chapter Three. In retrospect, the shortening of the incubation period seems to have been unnecessary as shown by the almost identical mean transfer constants found in the experiments of Chapter Three and those of the present experiments.

Theoretical objections to the use of the Lineweaver-Burk method: The use of an alternative method (Appendix I, and Table 5.1 II, brackets) shows that the parameters obtained with the present modification of the Lineweaver-Burk method are unsatisfactory estimates. The differences between the control values (Tables 5.3 and 5.7, section II and IV) confirm this impression and contrasts with the small standard deviations of the parameters A' , A'' , B' and B'' which give rise to statistical significances between numerically small differences. The poor fit of the model is probably due to the assumption that the intraexperimental variation alone is representative of the random error. This assumption arose from the earlier experiments on the uptake (Table 3.1) in which the interexperimental variation was not significantly greater than the intraexperimental. However, the range of concentrations of Li^+ was very different in the two series of experiments. Askelöf et al. (1976) examined the error structure of kinetic experiments. They found deviations from the usual assumptions about the variance of such experiments, especially if the range of concentrations of the independent variable was wide. The empirical formulae for the variance of the initial velocity arrived at by the authors require many replications; they also seem required primarily in the distinction between complicated models of enzyme activity. The object of the present experiments was to

examine evidence of saturation and some very simple models of inhibition. As the estimates by the more accurate method show a negative intercept in the experimental situation most likely to exhibit saturation in the uptake of Li^+ (see discussion of Chapter Four), it is very unlikely that the conclusions should be altered by the use of another statistical method.

Theoretical objections to the use of the Akedo-Christensen correction: Cohen (1975, p. 66 - 70) pointed out that the Akedo-Christensen plot cannot distinguish between a second component in the uptake showing simple diffusion and a second component showing concentrative uptake, but not showing saturation. However, Cohen seems to consider differences in concentration only, whereas the driving force in the passive uptake of electrolytes is the electrochemical potential. In principle, the correction factor, n , introduced by Christensen & Liang (1966) can be used in the calculation of transport constants in situations in which the concentration ratio at steady state between the cells and the medium is different from one; no assumptions are made about the nature of the concentrative uptake in its use.

The uptake at high concentrations of Li^+ was only measured during one incubation period (see Methods). Therefore, it had to be assumed that an extrapolation of the uptake of Li^+ to zero time would represent the same fraction of Li^+ in the medium in the presence of high and low concentrations of Li^+ . This assumption was justified by the fact that there were no systematic changes of the above fraction between 1 mM and 30 mM (Tables 5.1 - 5.8, corresponding values of col. (i) and (iv)). Also, it is unlikely that high concentrations of Li^+ should have caused any changes in the size of the extracellular space, as there were no changes in the swelling at high and low concentrations of Li^+ (Table 5.10).

Experimental objections to the use of the Akedo-Christensen

correction: The gradual decrease of $n' (=C_{cs}/C_{med})$ with increasing concentrations of Li^+ , mentioned earlier, might be due to saturation of a cellular structure or carrier, but the results of the kinetic experiments of the present chapter makes this unlikely. Pappius et al. (1958) measured the concentration of K^+ in cerebral slices, making up media with various proportions of Na^+ and Li^+ and at the same time keeping their joint concentration constant at 150 mM (l.c. Fig. 1). The effect of high concentrations of Li^+ in lowering the K^+ was pronounced. This is unlikely to be due merely to the low Na^+ in the medium because a similar experiment with choline by these authors had much less effect on the K^+ . Also, Kjeldsen et al. (1973), using 2 mM Li^+ in the medium, found the same concentration of K^+ in the slices with concentrations of Na^+ in the medium between 78 mM and 250 mM. Finally, Hillman & McIlwain (1961) showed that the membrane potential, which indicates the ratio of K^+ between the cells and the medium according to the Nernst equation, did not change before the Na^+ of the medium was below approx. 30 mM. In the present experiments, the K^+ of the slices was not measured so that the decrease in K^+ , indicating a low functional state of the slices at high concentrations of Li^+ , can only be inferred from the experiments of Pappius et al. (1958). The swelling, which usually also reflects the functional state of the slice (Chapter 2.1 i)), was unchanged in spite of high concentrations of Li^+ in the present experiments. However, this was also observed by Pappius et al., and thus is not incompatible with a low concentration of K^+ in the slices under such experimental circumstances. Hertz & Schou (1962) demonstrated a concentration dependent initial increase and a rapid decrease of the oxygen uptake in cerebral slices, using concentrations of Li^+ in the medium of approx. 10 mM to 200 mM (l.c. Fig. 9, 10 and 11). The dependence on metabolism of the Li^+ uptake was shown earlier in the present thesis (Table 2.6, Fig. 3.2 and Fig. 4.1). Therefore, it seems likely that high concentrations of Li^+ may in fact alter the

uptake of Li^+ itself. The discrepancy between the values of n at high concentrations of Li^+ and the results of Table 5.10 may be explained by differences in incubation times and also in concentration of Li^+ as shown by a steep increase in oxygen uptake between 50 mM and 100 mM (Hertz & Schou, Fig. 9). Consequently the assumption underlying the use of the Akedo-Christensen correction, namely that high concentrations of a solute have no other effect on the uptake than saturation, is unlikely to be true in the case of Li^+ .

Preliminary conclusions: The results of the present chapter must be interpreted with caution because two critical assumptions underlying the calculations, namely absence of interexperimental variation and absence of adverse effects of high concentrations of Li^+ are unlikely to be fulfilled. However, an improved estimate of some of the crucial data, and the almost identical rate constants in the presence of considerable changes in the Li^+ , K^+ and Ca^{2+} of the medium suggest that saturable mechanisms in the uptake of Li^+ cannot be essential for the interactions observed in Chapter Four.

SUMMARY

1. The possibility of a saturable uptake of Li^+ and inhibition of the uptake by K^+ and Ca^{2+} was investigated using concentrations of Li^+ 1 - 30 mM, K^+ 0 - 10 mM and Ca^{2+} 0 - 5 mM in the medium. Removal of the bias induced by the Lineweaver-Burk regression was attempted by means of statistical weights. The calculations showed no convincing evidence of saturation or inhibition.
2. From a hypothesis of a combination of saturable and passive uptake, correction for the passive contribution was attempted using Li^+ concentrations in the medium up to 120 mM. The results obtained are likely to have been confounded by adverse effects of these high concentrations of Li^+ .

3. Calculated rate constants for the initial uptake of Li^+ , 1 - 30 mM, were not significantly different in spite of the stated changes of the cation composition of the medium and not significantly different from the rate constants obtained with low concentrations of Li^+ (Chapter Three).
4. These results suggest that, within the experimental error, no saturable mechanisms are quantitatively important in the uptake of Li^+ in cerebral slices.

APPENDIX I: The weighted Lineweaver-Burk regression

Notations used:

<u>Symbol</u>	<u>Unit</u>	<u>Definition</u>	<u>Table</u>
C_{med}	mmol/l	concn. of Li^+ in the medium	5.1-5.8, col. (i)
β	mmol/(kg min)	true unidirectional influx of Li^+	
$\sigma^2(\beta)$		variance of β	
B	mmol/(kg min)	measured unidirectional influx of Li^+ (see Chapter 3)	5.1-5.8, col. (ii)
$s^2(B)$		estimated intraexperimental variance of B	5.1-5.8, col. (iii)
v	mmol/(kg min)	in eq. (1) initial velocity, in eq. (3) B after correction for passive uptake	5.1-5.8, col. (v)
w		statistical weight (see below)	
$S(xy)$		take the sum of $x y$	
x	l/mmol	$1/C_{\text{med}}$, independent variable in L-B regression	
y	kg min/mmol	$1/B$ or $1/v$, dependent variable in L-B regression	
B'	kg min/l	slope in L-B regression when $y = 1/B$	5.1-5.8, sect. II
B''	kg min/l	slope in L-B regression when $y = 1/v$	5.1-5.8, sect. IV
A'	kg min/mmol	intercept in L-B regression when $y = 1/B$	5.1-5.8, sect. II
A''	kg min/mmol	intercept in L-B regression when $y = 1/v$	5.1-5.8, sect. IV
K_m	mmol/l	B'/A' or B''/A''	
V_{max}	mmol/(kg min)	$1/A'$ or $1/A''$	

About the meaning of K_m and V_{max} in transport kinetics see e.g. Christensen (1975, p. 115 - 119).

The most widely used linear transformation of the Michaelis-Menten equation is that by Lineweaver & Burk (1934, eq. (4), notations changed): $1/v = K_m/(V_{\max} C_{\text{med}}) + 1/V_{\max}$ (1)

In the present experiments, the L-B regression was used in an attempt to estimate the kinetic parameters when Li^+ in the medium was 1-30 mM: $1/B = B'/C_{\text{med}} + A''$ (2)

and after correction for passive uptake (see Appendix II):

$$1/v = B''/C_{\text{med}} + A'' \quad (3)$$

Evaluation of K_m and $V_{\max}$ would only have meaning if the estimates of B' (or B'') were significantly different, and the estimates of A' (or A'') were positive and significantly different from null under the various experimental conditions investigated. However, as was demonstrated by simulation analysis, the Lineweaver-Burk regression distorts the parameters by giving undue emphasis to the smallest values of the velocity (Dowd & Riggs, 1965). As was also shown by the authors (l.c. Table II, lower part) the use of statistical weights could overcome the disadvantages of the Lineweaver-Burk method.

If β has a variance of $\sigma^2(\beta)$ then the variance of $1/\beta$ is $\sigma^2(\beta)/\beta^4$ (Wilkinson, 1961, p. 327, col. 1, notations changed) and the relevant weight is the inverse variance, $\beta^4/\sigma^2(\beta)$.

However, β and $\sigma^2(\beta)$ are unknown.

In the present calculations, the weight, $w = B^4/s^2(B)$ was used. It was based on estimates from single experiments.

The statistical expressions of Table 5.1 - 5.8, section II and IV are defined as follows:

$$\begin{aligned} x^2 &= S(wx^2) - (S(wx))^2/Sw \\ y^2 &= S(wy^2) - (S(wy))^2/Sw \\ XY &= S(wxy) - (S(wx))(S(wy))/Sw \\ \bar{x} &= S(wx)/Sw \end{aligned}$$

$$\bar{y} = S(wy)/S_w$$

$$s^2(A') = (1/S_w) + \bar{x}^2/X^2$$

$$s^2(B') = 1/X^2$$

$$s^2(A'') \text{ and } s^2(B'') \text{ are defined analogically}$$

From these expressions A' , B' , A'' and B'' (as well as K_m and $V_{\max}$) could be estimated. In the statistical comparison of the various slopes it had to be assumed that $s^2(B)$ and $s^2(B'')$ were known exactly with an infinite number of degrees of freedom. Consequently the usual multiple range test (Duncan, 1955) was not preceded by the analysis of variance.

Alternative method (Newman, 1979, personal communication):

If the variance of B_i , $\sigma^2_{B_i}$, at each level of C_{med} is known and small compared with the expected value of B_i , β_i , then the variance of $1/B_i$ is σ^2/β^4 .

Defining x and y as shown in Notations, and assuming that the linear function $A' + B' x_i$ gives the expected value, $\hat{y}_i$, of y_i at level x_i , then the variance of $\hat{y}_i$ is $\hat{y}_i^4 \sigma^2_{B_i}$.

In the method used, A' and B' are determined as the values that minimize E in the expression:

$$E = S(y_i - \hat{y}_i)^2 / \hat{y}_i^4 \sigma^2 \quad (4)$$

This is done by letting dE/dA' and dE/dB' equal zero and solving the equation for A' and B' . The equations have to be solved by iteration. Using estimates of A' and B' calculated by the previous method (Table 5.1, section II) as the first trial values, a reasonable fit was reached after five iterations (l.c., brackets). The difference between the first and final estimates demonstrates that the first estimate is far from satisfactory.

APPENDIX II: The Akedo-Christensen correction.

<u>Symbol</u>	<u>Unit</u>	<u>Definition</u>	<u>Table</u>
C_{med}	mmol/l	concn. of Li^+ in the medium	
C_{CS}	mmol/kg	concn. of Li^+ in cerebral slices after time, t.	
B	mmol/(kg min)	measured unidirectional influx of Li^+	5.1-5.8, col.(ii)
$s^2(B)$		estimated intraexperimental variance of B	5.1-5.8, col.(iii)
v	mmol/(kg min)	B after correction for passive uptake	5.1-5.8, col.(v)
$s^2(v)$		calculated variance of v, see below, and eq(8) to (10)	
V_{max}	mmol/(kg min)	$v = V_{max}$ when the saturable uptake is maximal	
A	mmol/kg	$C_{CS} - Bt$; $t = 1-4$ min	5.1-5.8, col.(iv)
a_i		A/C_{med} ; $C_{med} = 1-30$ mM	
$\bar{a}$		$\text{Sum}(a_i)/N$; $N = 8-10$ exps.	5.1-5.8, sect.III
n		C_{CS}/C_{med} after $t = 60$ min and $C_{med} = 120$ mM	5.9, col.(ii)
n'		value of n when $C_{med} < 120$ mM	5.2;5.3;5.6; 5.7,col.(vi)
d		$= 1 - e^{-k_D t/n}$; $t = 2.33$ min, non-saturable, membrane-limited diffusion	5.9, col.(iii)
k_D	min^{-1}	Rate constant of d	5.9,col.(iv)
c	min^{-1}	k_D/d , correction factor for zero order uptake	
$\bar{a}/n$		the uptake of Li^+ at $t = 0$ as a fraction of the uptake at $t = 60$ min	

<u>Symbol</u>	<u>Unit</u>	<u>Definition</u>	<u>Table</u>
* $(dv/dB)^2$		correction factor for calculation of s^2 (v) from $s^2(B)$, eq (8) to (10)	5.9, col. (v)
p		$n(d + \bar{a}/n)$	5-9, col. (i)

* In this context 'd' is the differential notation.

The combination of a saturable and a non-saturable process was investigated by Akedo & Christensen (1962) in the uptake of amino acids, and a kinetic equation was derived that made it possible to correct for the non-saturable component (1.c p. 119, eq 2). The equation was modified for substances with ratios of concentration across the cell membrane different from one (Christensen & Liang, 1966; p. 5554). In the present experiments, it was necessary to allow for the uptake at zero time (Fig. 3.1 and Table 5.1 - 5.8, col. (iv)) which could not be observed at high concentrations of Li^+ because there was only one incubation period, but which had to be assumed in order to compare the uptakes at high and low concentrations. With these two modifications, and change of notations Akedo & Christensen's eq 2 can be written as follows:

$$C_{CS}/(nC_{med}) = v/(cC_{med}) + (d + \bar{a}/n) \quad (5)$$

At high concentrations of Li^+ , the saturable uptake is assumed to be saturated so that v becomes constant ($= V_{max}$). This is the basis of the graphical determination of d (here: $d + \bar{a}/n$) as the intercept of a straight line relating $C_{CS}/(nC_{med})$ to $1/C_{med}$ at various high concentrations (e.g. Winter & Christensen, 1964, Fig. 7). However, the intercept was difficult to determine in this way because of experimental variation. Consequently, the equation was rearranged:

$$C_{CS} = (d + \bar{a}/n)nC_{med} + V_{max}n/c \quad (6)$$

From this expression $(d+\bar{a}/n)n$ could be calculated as the coefficient of a straight line with C_{med} ($= 40-120 \text{ mM Li}^+$) as the independent variable, and the usual distortion of a reciprocal plot was avoided (see Appendix I). The results are shown (Table 5.9 (i)). The estimates of n were measured in separate experiments (Table 5.9 (ii)). Hence d and $k_D (=c/d$, see definitions) could be calculated (Table 5.9, column (iii) & (iv)).

From eq (5):

$$v = (C_{\text{CS}}/n - (dC_{\text{med}} + \bar{a}C_{\text{med}}/n))c$$

As $C_{\text{CS}} = Bt + A$, and $A = a_i C_{\text{med}}$, eq (5) could be simplified considerably and some of the experimental variation reduced by assuming $a_i = \bar{a}$. Thus the simplified expression:

$$v = (Bt/n - dC_{\text{med}})c \quad (7)$$

could be obtained, which was used in the calculation of v (Tables 5.1 - 5.8, column (v)).

The expressions in eq (7) are determined experimentally (Table 5.9) and the variation of their values must be taken into account in the variance of v . The full expression for calculating the variance of v is complicated, but several terms contribute little to the variance and can be ignored under the present circumstances, leaving the simplified expressions (Newman, 1979, personal communication):

$$s^2(v) = (dv/dB)^2 s^2(B) \quad (8)*$$

$$= (Q^2/(P - \bar{a})^2) s^2(B) \quad (9)$$

$$Q = n(\log_e n - \log_e (n - (P - \bar{a}))) \quad (10)$$

in which $\bar{a}$, n and P are experimental values defined in Notations and shown in Table 5.1 - 5.9. The correction factor that must be multiplied with $s^2(B)$ is shown (Table 5.9, col. (v)) and can be considered constant for any one table.

* In this context 'd' is the differential notation.

THE EFFLUX OF LOW CONCENTRATIONS OF Li^+ FROM CEREBRAL SLICES

The experiments of Chapter Three and Four demonstrated that the uptake of Li^+ in cerebral slices was concentrative, that it was dependent on metabolism, and that there was interaction with K^+ in the uptake.

The aim of the experiments of this chapter was, first to perform a compartmental analysis on the basis of longer observations than those of Chapter Three, secondly, to compare the rate constants of the unidirectional influx and efflux and, if possible, apply criteria for active transport, and thirdly, to see whether an apparent inhibition by Li^+ of the K^+ uptake (Table 4.2) would be reversible.

METHODS

Preparation of tissue and incubation: Cerebral slices were prepared as described earlier, except that they were not weighed before incubation.

They were not pre-incubated because the duration of the incubations was longer than in the earlier experiments, and it was anticipated that the functional activity of the slices might decrease if the incubations were prolonged any further. This omission made no difference in the concentration of Li^+ at steady state (see Results).

After 1 h incubation in a standard medium (Table 2.2) to which had been added Li^+ 1.5 mM, the slices were taken up with a bent rider (Fig. 2.2), passed quickly through a Petri dish with a medium without Li^+ , and incubated in a standard

medium of 10 ml without Li^+ .

The slices were taken out after 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 120 and 150 min. They were drained and weighed as described earlier.

Measurement of Li^+ : The sensitivity of the Li^+ measurements (Chapter 2.2 iv)) was not high enough to allow estimation of the efflux on the basis of the Li^+ transferred from the slices into the medium. Therefore, it was necessary to use one slice at each incubation time in each experiment. Eight experiments were used to construct the efflux curve. In order to reduce the interexperimental variation, the concentrations (C_{cs}) measured in each experiment were related to the concentrations ($C_{\text{cs}\infty}$) in 4 - 6 of the slices incubated for 1 h in the presence of 1.5 mM Li^+ without being transferred afterwards (Fig. 6.1).

Li^+ , K^+ and Na^+ were measured as described earlier. Volumes of 6% TCA from 0.7 ml to 2.5 ml were used for the homogenization.

Calculations: The experimentally determined curve showing the efflux was plotted semilogarithmically and separated into monoexponential functions by the residual method, as described e.g by Solomon (1960). In an appendix to that chapter, a method for correction of the intercepts is shown. As mentioned in the discussion of Chapter Three the kinetics of a fast component may influence those of a slow one. This happens if the compartments form a serial system like the intracellular and extracellular space in a cerebral slice. If the size of the spaces and the rate constants do not differ much the estimates of the size of the compartments may be considerably in error. The correction of the intercept is as follows: $S' = FS(k_f - k_s)^2 / (Fk_f^2 + Sk_s^2)$, in which F and S are the fast and slow compartment, respectively, and k_f and k_s are the corresponding rate constants in the two monoexponential functions, $Fe^{-k_f t} + Se^{-k_s t}$, describing the observed efflux

curve (Huxley, 1960, p. 165, eq B 8, notations changed).

Determination of the slow component in the curve was based on the last six points (Fig. 6.1, circles). A straight line was fitted by the method of least squares. This line was used in the subtraction by which the points of the fast component were calculated (Fig. 6.1, squares). No statistical weights were applied.

RESULTS

Li⁺ at steady state: Omission of the pre-incubation in a standard medium without Li⁺ had no effect on the results. The concentration of Li⁺ in the slices incubated for 1 h with 1.5 mM Li⁺ was 2.113 mmol/kg $\pm$ 0.195 (mean $\pm$ S.D. of 8 estimates, 34 slices); this was not significantly different from all earlier estimates (Table 2.6a). In a separate experiment, slices were incubated in the presence of 1.5 mM Li⁺ for up to 180 min without showing any significant changes from the 1 h value (results not shown). This was taken as an indication that the efflux took place from a situation which was comparable to earlier experiments at steady state of Li⁺ in the slices.

Efflux of Li⁺: The mean values of eight experiments are shown (Fig. 6.1). Under these experimental circumstances, the curve could be resolved into two monoexponential functions with reasonably good fits of the points, especially of the fast component, in which the measurements were more reliable. The rate constants of the fast and slow component were $(7.16 \pm 1.78)10^{-2} \text{ min}^{-1}$ and $(1.56 \pm 0.42)10^{-2} \text{ min}^{-1}$ (mean $\pm$ S.D. of 8 estimates, 121 slices); this corresponded to half-times of 9.7 min and 44.5 min. The intercepts of the fast and slow components were $68.2\% \pm 13.5(8)$ and $14.1\% \pm 4.2(8)$, respectively.

Huxley's correction: Correction of the intercepts requires knowledge of the size of the extracellular space and the rate

of diffusion of Li^+ in this space, none of which were measured in the present experiments. As was done earlier (Chapter Three) the extremes of 20% and 40% were used as well as the corresponding values of the calculated half-times and rate constants; these were 21 s ($k = 1.98 \text{ min}^{-1}$) and 17 s ($k = 2.45 \text{ min}^{-1}$), respectively. Furthermore, the correction requires an interpretation of the slow component. If this component does have an anatomical correlate, this may be cellular or sub-cellular. A cellular location would require correction for extra-cellular diffusion, and a sub-cellular location would place the slow compartment in series with the fast, "cellular" compartment. In the first case, the slow component is unaffected (13.9%) for both sizes of the extra-cellular space, whereas the fast component is slightly reduced (63.1% or 64.2% if the extracellular space is 20% or 40%, respectively). In the second case, correction by means of the fast, cellular compartment reduces the slow compartment to 8.5% and increases the fast compartment correspondingly (to 73.8%). However, this compartment is again reduced by the presence of the extracellular space to its former size (68.2% or 69.4% if the extracellular space is 20% or 40%, respectively). It is obvious then, that the assumptions about the extracellular space are not critical and that the corrections are almost negligible whatever the interpretations.

K^+ and Na^+ during efflux: The concentration of K^+ in the slices showed no indication of interaction with Li^+ in the present experiments (Table 6.1). After 1 h incubation, the K^+ was the same, whether Li^+ was present in the medium or not. After 2 h incubation, the K^+ had fallen significantly, whether Li^+ was leaving the slices or not. The concentrations of Na^+ were unchanged in the presence and absence of Li^+ and were not dependent on the incubation time within the observation period.

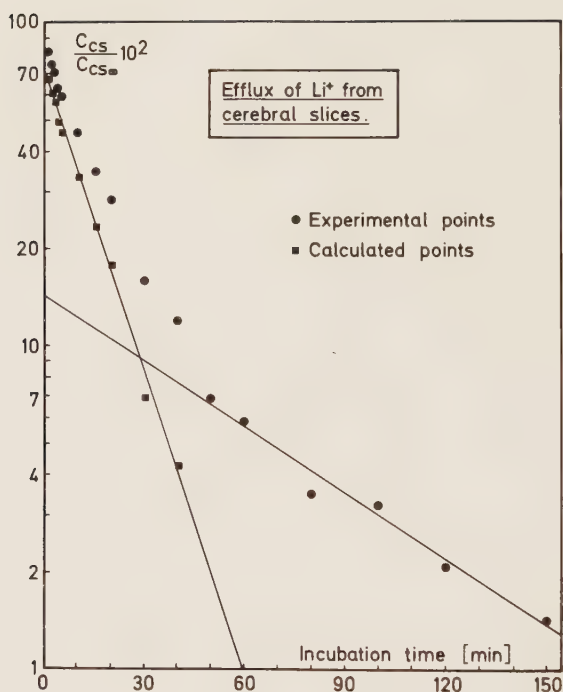


FIG. 6.1

Efflux of Li^+ . The slices were incubated for 1 h (without pre-incubation) in a standard medium also containing Li^+ 1.5 mM. They were then transferred to a standard medium without Li^+ , and Li^+ in the slices was measured at various intervals between 1 and 150 min, as indicated. Each experimental point, ●, represents the mean of 6 - 8 slices. In each of 8 experiments Li^+ was measured in 4 - 6 slices after 1 h incubation with Li^+ and the mean concentration was used as $C_{cs\infty}$ for that experiment. The methods and results of the calculations are described in the text.

TABLE 6.1 EFFECT OF Li^+ AND INCUBATION TIME ON K^+ AND Na^+ IN CEREBRAL SLICES

Procedure	(a) UPTAKE (1 h)	(b) CONTROL (1 h)	(c) EFFLUX (1 h)	(d) CONTROL (2 h)
K^+ concn (mmol/kg)	$58.6 \pm 6.2(10)$	$58.7 \pm 5.1(10)$	$49.2 \pm 4.9(10)$	$53.4 \pm 3.7(9)$
Na^+ concn (mmol/kg)	$103.6 \pm 5.6(10)$	$104.1 \pm 5.6(10)$	$106.0 \pm 4.0(10)$	$106.8 \pm 5.4(9)$

Cerebral slices were incubated in standard media in the presence (column a) or absence (column b and d) of Li^+ 1.5 mM for the times indicated. In c) the slices were first incubated for 1 h in the presence of Li^+ 1.5 mM and then transferred to a non- Li^+ medium as was done in the efflux experiments. Values are the means $\pm$ S.D. of the number of slices in brackets; they are the combined results from two experiments. The missing value in(d) was replaced according to Bliss (1967, p. 316-318) prior to a two-way analysis of variance. In the K^+ concentrations, there was a highly significant effect of the incubation time ($p < 0.001$). None of the other comparisons were significantly different; in particular, there was no interaction between Li^+ and incubation time.

DISCUSSION

The fit of the model: There are no criteria for the selection of points in the resolution of a kinetic curve by the residual method, except that they should fit a straight line. In the presence of experimental variation, there is the problem, mentioned in Chapter Three, on the one hand, of getting a sufficient number of points for a reliable regression analysis, and on the other hand, of inducing systematic errors by the use of too many points.

A priori, the four last points of the curve might be as good as the six that were chosen. This choice would change the slow component somewhat (intercept 11%, $T_{1/2}$ 51 min), whereas the fast component would be less affected (intercept 75%, $T_{1/2}$ 9.3 min). The correlation of the fits was practically the same. However, the calculated points of the fast component clearly showed systematic deviations from the fitted line, whereas the present points (Fig. 6.1, squares) can be seen to be naturally distributed. This indicates that the present choice of points is reasonable.

The slow component: The efflux of Li^+ was observed in a closed system, as the slices were not transferred during the incubation in the Li^+ -free media. Therefore, in principle, the efflux was not unidirectional. However, the volume of the slices was approx. 1/200 of that of the medium. Although two slices were usually incubated together, they were paired in such a way that slices with long and short incubation times were incubated together. Actual measurements of Li^+ in the media after efflux showed concentrations which were less than 2% of the concentrations at steady state. This means that the lowest values of the efflux curve are almost in a new steady state with the Li^+ of the medium, corresponding to the situation of the net influx (Fig. 3.2). Although this would alter the rate constants by approx. 2%, it could not cause a deviation from linearity and hence it could not

be the reason for the slow component.

The fact that only one component was seen during the influx (Fig. 3.2) does not exclude an anatomical counterpart of the slow component, because the observation period was also longer. With a half-time of 45 min it would require 200 min ($4-5 \times T_{1/2}$) to approx. equilibrium. However, no significant change in the concentration was observed up to 180 min incubation in the presence of Li^+ , as was mentioned before, nor was there any indication of systematic, non-significant increases.

The long incubation time might have another effect which would not be observed during the influx, namely a change in the functional state of the slices. This was indicated by the decrease of the concentration of K^+ in the slices during incubation (Table 6.1). In the absence of oxygen a half-time of the influx of 37 min was observed (Fig. 3.2). This is not significantly different from the half-time of the slow component. As the uptake was not concentrative during anoxia, (Table 2.6b), the rate constants of uptake and efflux should be alike under these circumstances. Thus the slow component could represent a population of damaged cells. As was discussed earlier (Chapter 2.1 A ii)b) experiments have shown considerable inhomogeneity in the functional state of the cells in cerebral slices, both biochemically and microscopically (Cohen, 1974b; Møller et al., 1974; Sershen & Lajtha, 1974).

Fourteen per cent may seem a small proportion of damaged cells, considering the low K^+ values. However, assuming a Li^+ concentration of the damaged cells near that of the medium, the lithium associated with the compartment represents $14 \times 1.4 = 20 \mu\text{l}$ per 100 mg incubated wt. (see calculation of extracellular space below) and thus 35 - 40% of the total volume of $53 \mu\text{l}$ per 100 mg incubated wt. arrived at later. The question of cell damage was not investigated further, but it would seem the most likely explanation of the slow component.

The fast component: The half-time of this component was approx. 10 min. This is 20 - 30 times longer than the half-time calculated for the extracellular diffusion of Li^+ (17 - 21 s, see Chapter Three). This, and the size of the compartment makes it likely that it represents membrane limited diffusion.

Estimate of the extracellular space: The extracellular diffusion of Li^+ is too fast to be recorded with the present techniques. However, an estimate is possible from the values of the 'cellular' compartments. As was shown earlier, the sum of the slow and fast component remains almost the same whatever the assumptions about the extracellular space. Using 77% as a mean estimate of Li^+ located in these compartments, 23% of the Li^+ is in the extracellular space and adhering fluid at steady state. In the present experiments the concentration ratio of Li^+ between the tissue and the medium was 1.4. In order to compare the estimate with the conventional way of presenting the inulin space, the swelling must also be corrected (approx. 20%). This results in an estimate of $23 \times 1.4 \times 1.2 = 39 \mu\text{l}$ per 100 mg fresh wt. Correction for adhering fluid not removed by quick passage, approx. 10% (Lund-Andersen, 1974), results in an estimate of approx. $30 \mu\text{l}$ per 100 mg fresh wt. Except for the corrections for adhering fluid, the corrections are based on measurements under the present experimental conditions. The mean value of approx. 30% is very near those found from the steady state efflux kinetics of inulin (Lund-Andersen, 1974; Møller et al., 1974).

Comparison of k_{in} and k_{out} : The transfer constant for efflux, k_{out} , was not significantly different from the transfer constant for influx, $k_{\text{in}} = (8.75 \pm 0.65) 10^{-2} \text{ min}^{-1}$, $n = 6$, calculated in Chapter Three. The uptake was concentrative with a steady state ratio of Li^+ between the tissue and the medium of 1.4 in the present experiments. By definition, the influx and efflux are equal at steady state. Therefore, k_{out} , should be less than k_{in} at steady state according to the

equation $k_{in} = k_{out} \times C_{cs}/C_{med}$ (Harris & Maizels, 1951, eq (4), notations changed). How much less, would depend on the intracellular activity. Using the value of $23 \times 1.4\mu\text{l} = 32\mu\text{l}$ per 100 mg incubated wt. for the extracellular space and adhering fluid, and a dry wt. of 15 mg per 100 mg incubated wt., an intracellular space of $53\mu\text{l}$ per 100 mg incubated wt. is arrived at. This would result in a concentration ratio of 2. If the slow component represents damaged cells with a concentration approx. like that of the medium, these would have a volume of $20\mu\text{l}$, increasing the concentration ratio of Li^+ between the remaining cells and the medium to 2.7. Provided that activity equals concentration, k_{out} should then be, at most, half as great as k_{in} at steady state. The reasons for this discrepancy may be (1) intracellular concentrations of Li^+ are different from their activities, (2) the fluxes are not representative of the steady state, (3) errors in the measurements of the constants.

(1) As mentioned, the slow component may represent Li^+ located sub-cellularly, but in that case Huxley's correction would reduce the size to 12% of the fast compartment, and consequently it would diminish the intra/extracellular activity ratio very little. However, association with sub-cellular structures need not be detectable in kinetic studies if the rate of release is equal to or higher than that of the fast component and thus we cannot predict the activity from the efflux studies. The affinity studies with homogenates and fixed slices (Chapter 2.1B iv) and v)) did not give a clear result, but the finding that anoxic slices had concentrations near those of the medium makes a high affinity of the tissue for Li^+ unlikely. Activity measurements with Li^+ electrodes have been performed on snail neurons recently, (Thomas et al. 1975); the authors demonstrated an intra/extracellular activity ratio less than one with external concentrations from 1 mM to 10 mM. However, until measurements on excitable tissue from other

animals are reported, species differences cannot be excluded.

(2) As tracer studies of the kinetics of Li^+ are not practicable, the flux measurements were not performed at steady state. However, this is not a condition for comparability of influx/efflux. Harris and Maizels (1951) compared flux measurements of Na^+ in red cells by means of 'chemical' methods like the present, and by means of radioactive tracers at the steady state; the results obtained by the two methods were in reasonable agreement (l.c. Table p. 519). Nevertheless, cerebral slices are more vulnerable to change than red cells. In order to be representative of the steady state (a) the transfer constants should be determined during unidirectional flux, (b) systems of importance to the transfer of Li^+ should not be modified during the measurements, and (c) none of the transfer systems should require Li^+ on both sides of the membrane, nor should any modification by Li^+ be dependent on the time of exposure.

(a) The determination of unidirectional influx has been discussed in detail (Chapter Three). Clearly, the influx was not unidirectional during the whole period of uptake; the efflux was (almost) unidirectional.

(b) Significant changes in the tissue during 5 min influx are unlikely; during efflux the tissue did change as shown by the K^+ values (Table 6.1); the fast component may represent undamaged tissue, but this was not tested experimentally.

(c) As was summarized (Chapter Four) the three important modes of transfer of Li^+ in red cells, and hence possibly in cerebral cells are i) passive transfer 'leak diffusion', ii) the Li-Na^+ countertransport system, iii) the K-Na -activated sodium-pump. i) If Li^+ caused a decrease in the membrane potential Li^+ influx would decrease. However, according to the Nernst equation, shown later, this would also cause a decrease of the K^+ in the slices. No effect of

Li^+ on the K^+ was seen in the present experiments (Table 6.1). ii) As discussed later (Chapter Eight), the Li-Na^+ countertransport system depends mainly on the concentration ratio of sodium between the cells and the medium. The concentration in the medium was constant and that in the slices was not changed by Li^+ or incubation time in the present experiments (Table 6.1). iii) As discussed in Chapter Four the effect of low concentrations of Li^+ on this system is small in a standard medium.

(3) Errors in the measurements of k_{in} and k_{out} are difficult to separate from the problems of the previous section. Back diffusion would make the rate constants too low and influence from extracellular diffusion would make them too high; omission of the dry wt. in the calculations was common to both constants, while the average cell volume is likely to have changed only during the efflux. As was discussed earlier (Chapter Three) the tendency was slightly in favour of too high values of k_{in} and, therefore, cannot explain the discrepancy. Back diffusion in the efflux experiments was negligible and influence from the extracellular space was small, judged by the effect of Huxley's correction. Assuming the same amount of Li^+ in the cells, cellular swelling would lower the concentration and thus the apparent rate of efflux, whereas k_{out} was in fact higher than expected.

Thus it cannot be said with any certainty which of the factors (1) - (3) are responsible for the equality of the two rate constants.

Criteria of active transport: The Nernst equation relates the membrane potential π (negative) and the outside and inside activity of a monovalent cation $A^+_{\text{out}}/A^+_{\text{in}}$ at 37°C as follows: $\pi = 61 \log (A^+_{\text{out}}/A^+_{\text{in}})$. Assuming a mean potential difference between the cells of a cerebral slice and the medium of 61 mV, the equation would predict an intra/extracellular activity ratio of 10 if Li^+ is distributed according

to its electrochemical potential. Provided that activity equals concentration this is far from the case according to the previous calculations. Therefore, the cerebral cells may not be in an electrochemical equilibrium at steady state of the Li^+ concentrations because of active extrusion of Li^+ . According to Ussing, the ratio between the influx and efflux should equal the ratio of electrochemical activities of the ion in the outside solution and the inside solution if the ion moves passively through a membrane (Koefoed-Johnsen & Ussing, 1960, eq (6) p. 173). Ussing's flux ratio need not be measured at steady state, but the membrane potential should be measured under conditions comparable to the flux measurements. This was not done in the present experiments. Also, as mentioned above (1) - (3), there are too many uncertainties in the flux measurements for a comparison, even if one were to use values for membrane potentials from the literature (e.g. Li & McIlwain, 1957, Hillman & McIlwain, 1961). Therefore, it cannot be concluded from the present experiments, whether Li^+ is extruded actively from the cerebral slices. The experiments by Thomas et al. (1975) showed transport of Li^+ against an electrochemical gradient from snail neurons. Araki et al., (1965) injected cations into cat spinal motoneurons by iontophoresis and showed that the rate of extrusion of Li^+ was about half that of Na^+ . Most other authors have found very slight or no active extrusion of Li^+ from nerve and muscle preparations (Keynes & Swan, 1959; Baker, 1965; Gardner & Kerkut, 1968; Wespi, 1969). Species differences, various states of functional activity of the preparations and various concentrations of Li^+ in a saturable system might explain the different findings.

K^+ and Na^+ during efflux: In the experiments of Chapter Four there were some indications of lowered K^+ values in the slices in the presence of low Li^+ concentrations in the medium, confirming previous findings (Kjeldsen et al., 1973). The present experiments were carried out to see whether this ap-

parent inhibition of the K^+ uptake would be reversible, i.e. whether removal of Li^+ from the slices would increase the K^+ concentrations of the slices. On the contrary, the K^+ decreased during the incubation in Li^+ free media and removal of more than 90% of the Li^+ of the slices (Table 6.1; Fig. 6.1). Furthermore, there was no indication of a decrease of the K^+ of the slices in the presence of Li^+ for 1 h. The K^+ values in the absence of Li^+ were low compared to those of Kjeldsen *et al.*, and, as was discussed in Chapter Four, it seems likely that difference in the functional state of the slices might explain the discrepancies. Deterioration of the slices in the present experiments might override any tendencies in the opposite direction.

SUMMARY

1. The efflux was measured up to 150 min after 1 h incubation in a standard medium also containing Li^+ 1.5 mM. Under these experimental conditions the efflux was compatible with two compartmental first order kinetics. The half-times of the fast and slow components were 9.7 min and 44.5 min, respectively, and their corresponding compartments made up 68% and 14% of the concentration of Li^+ in the medium. Huxley's correction altered the size of the compartments negligibly.
2. The fast component was interpreted as representing membrane limited efflux because of its rate and the size of its compartment. The slow compartment was found likely to represent efflux from damaged cells.
3. The experimental technique did not allow measurement of the diffusion of Li^+ from the extracellular space of the slices. However, an indirect estimate of the extracellular space of 30 μ l per 100 mg fresh wt. is close to published values obtained by reliable techniques.
4. In spite of the unequal distribution of Li^+ between the tissue and the medium, the rate constants of influx and ef-

flux were not significantly different. Possible reasons for this are discussed.

5. Though extrusion of Li^+ from the slices is likely, the data do not permit application of established criteria for active transport.

6. The concentration of K^+ of the tissue during efflux of Li^+ suggests a low functional activity of the slices after the necessary long incubations.

THE KINETICS OF Li^+ IN THE BRAIN, CEREBROSPINAL FLUID AND
SERUM OF THE RAT

It has been shown in rats that when the Li^+ concentration in the serum changes rapidly, it does not correlate well with that found in the brain (Frazer *et al.*, 1973). Paul *et al.* (1973) and Watanabe *et al.* (1973) found a high correlation at steady state between the Li^+ concentrations in the serum and in the cerebrospinal fluid (CSF). These authors assumed that the concentration in the CSF would reflect the cerebral concentration better than that found in the serum.

The aim of the following experiments was to compare directly in rats the concentration of Li^+ in the serum, the CSF and the brain at steady state and also to examine the relationship between these concentrations during the uptake and elimination of Li^+ . These data could be compared with the findings in patients and in the previous experiments on cerebral slices.

METHODS

Administration of Li^+

Male Wistar albino rats weighing 250 g - 400 g were used.

(1) For measurement of Li^+ at steady state, 8 rats were given a powdered standard diet (Spratt's No. 2) for 4 days followed by a further period of 4 days during which they were given a similar diet to which lithium carbonate (55 mmol Li^+ /kg dry food) and sodium chloride (300 mmol/kg dry food) had been added. (2) In one series of kinetic experiments a single intraperitoneal injection of lithium chloride (500

mmol/l, 5 mmol/kg body wt.) was given to 35 rats. (3) In a second series of kinetic experiments 56 rats were given subcutaneous injections of lithium chloride (150 mmol/l, 0.9 mmol/kg body wt.) every 6 h for 2 days, corresponding to approx. 8 half-lives ($T_{1/2}$). Use of subcutaneous administration of lithium chloride to rats has been shown to give a more complete absorption than the oral route and to avoid the high peaks in the serum concentration resulting from intraperitoneal injections (Olesen *et al.*, 1976, Fig. 1). In (2) and (3) the rats were given pellets of the above standard diet without any additions and had access to a solution of sodium chloride (155 mmol/l) during and after the administration of Li^+ . The addition of extra sodium chloride to the diet, or access to a sodium chloride solution has been shown to prevent intoxication during administration of lithium to rats (Thomsen & Olesen, 1974). The rats were allowed to drink water ad libitum.

Preparation of tissue: All rats were anaesthetized with ether, and blood samples of approx. 5 ml were taken by cardiac puncture and centrifuged without the addition of anti-coagulants. Samples of CSF were drawn from the cisterna magna by the technique described by Smith & Balagura (1972), slightly modified by the use of a disposable tuberculin syringe connected by tubing to a mouthpiece. By slight suction it was possible to take samples of approx. 0.1 ml, which increased the sensitivity of the measurements. The samples were transferred to pre-weighed tubes and weighed immediately afterwards. Samples that were blood stained were discarded. The necks of the rats were then dislocated and the brains were rapidly removed and placed on moist filter paper in a petri dish.

In (1) the rats were killed after 4 days on the Li^+ diet. In (2) the rats were killed from 0.25 h to approx. 48 h after the injection, and in (3) 6 h to approx. 48 h after the last injection.

All samples were stored at 4° for a maximum of 5 days before the measurement of lithium. Then the serum was diluted by 6 to 150 times with a solution of sodium chloride (155 mmol/l), and 0.7 - 1.2 ml of this solution was added to all the samples of CSF. The brains were blotted with filter paper, and specimens of whole brain, weighing 100 mg - 800 mg, were homogenized in 2 - 4 ml of 6% TCA.

Measurement of Li⁺

Li⁺ was measured as described earlier (Chapter 2.2). In addition to the standards with TCA for the cerebral tissue mentioned there, standards for the measurement of lithium in the serum and CSF were made up in sodium chloride (155 mmol/l), as the latter standards had been shown in recovery experiments to give reproducible results with all the dilutions used in the present experiment. Double determinations were made on the samples of serum and cerebral tissue, the relative standard deviations being 2% and 5% respectively; the samples of CSF were too small for double determination.

As lithium chloride solutions were acid (Chapter 2.2i)), they were neutralized with sodium hydroxide pellets, if they were to be administered to rats.

Calculations: Concentrations of Li⁺ are referred to the wet weight of the cerebral tissue or to the total volumes of the CSF or serum. The statistical methods were described earlier (Chapter 2.4).

RESULTS

Fig. 7.0

Steady state (Table 7.1/): The Li⁺ concentrations measured in the cerebral tissue, CSF and serum after oral administration of lithium carbonate for 4 days are shown together with the corresponding ratios between these values. There was no significant difference between the concentrations in the brain and in the serum, but that of the CSF was almost one third of

these values (Table 7.1 i)). There were considerable inter-individual differences in the concentrations, whereas the intra-individual ratios between the concentrations showed smaller variations (Table 7.1 ii)). Linear plots of Li^+ in the cerebral tissue and CSF relative to Li^+ in the serum had correlation coefficients of 0.93 and 0.96, respectively (Fig. 7.0).

Single injections (Fig. 7.1): The concentration of Li^+ in the brain slowly reached a maximum of approx. 1.5 mmol/kg between 9 h and 24 h. In the CSF, the maximum concentration of 1.2 mmol/l was seen between 45 min and 2 h. Between 15 min and 2 h the concentration of Li^+ in the CSF was greater than that of the brain. Serum concentrations were maximal, approx. 8 mmol/l when the first sample was taken after 15 min.

The elimination of Li^+ from the brain, CSF and serum took place at different rates even from 24 h to 50 h, when the half-life was found to be 12 h, 10 h and 7 h, respectively. From the kinetic curve (Fig. 7.1) it may be difficult to determine when the elimination became linear, because of the rather large variation. Relating the concentrations of Li^+ in the brain and in the CSF to the corresponding concentrations in the serum reduced the scatter (Fig. 7.2). From this figure is seen that the ratios of concentrations of Li^+ in the CSF and serum, and in the cerebral tissue and serum at steady state (Table 7.1) were reached between 10 h and 20 h, but the ratios continued to rise until an apparent equilibrium was reached around 40 h. However, only the elimination of Li^+ in the serum could be shown to be linear from 40 h to 50 h ($T_{1/2} = 12$ h, $r = 0.90$, $N = 6$), while the corresponding fitted regression lines for the elimination of Li^+ from the CSF and the cerebral tissue were not statistically significant.

Elimination from steady state (Fig. 7.3): The steady state levels in the cerebral tissue, serum and CSF, obtained after

repeated subcutaneous injections, as described in methods, were 0.9 mmol/kg, 0.8 mmol/l and 0.3 mmol/l respectively, as estimated from the first three sets of concentrations from 6 h to 7 h after the last injection. From then on, elimination from the three compartments took place at different rates. Also in this experiment the points on the kinetic curve showed a large variation. Therefore, as in the previous experiment, the concentrations of Li^+ in the brain and in the CSF were related to the corresponding concentrations in the serum in order to get a more precise impression of the changes (Fig.-7.4). The initial values were very close to the values at steady state from the feeding experiment (Table 7.1) ^(Fig.7.0) 7. Thereafter the ratios increased until approx. 24 h when a fitted regression line was no longer significantly different from zero, indicating that a new equilibrium had been reached. The corresponding joint ratio between the concentrations in the cerebral tissue and serum was 4.71 ± 0.28 , and in the CSF and serum was 0.773 ± 0.032 (mean $\pm$ s.e. mean of 25 and 21 ratios respectively).

The calculated elimination constants before and after 24 h are shown (Table 7.2). The elimination rate of Li^+ in the serum slowed down, that of the brain increased, and that of the CSF remained unaltered. After 24 h the constants were no longer significantly different, indicating that the β -phase of elimination had been reached.

DISCUSSION

In the present experiments the concentration of Li^+ after single injections was higher in the CSF than that of the brain for approx. 2 h. These results are representative of average cerebral tissue, although different rates of uptake of lithium in various areas of the rat brain have been observed under similar experimental conditions (Mukherjee et al., 1976). The bulk of evidence shows that the blood-CSF and blood-brain barriers are similar with respect to the permeability

TABLE 7.1 STEADY STATE CONCENTRATION OF Li^+ IN THE BRAIN,
CSF AND SERUM

	(a) Brain	(b) CSF	(c) Serum	<u>P</u> value of differences
i) Concentration	0.482 $\pm$ 0.043 (8)	0.172* $\pm$ 0.018 (7)	0.435 $\pm$ 0.030 (8)	< 0.01
ii) Relative to serum con- centration	1.114 $\pm$ 0.033 (8)	0.407 $\pm$ 0.013 (7)	1	< 0.001

Values are expressed as mmol/kg of wet cerebral tissue or as mmol/l of serum or CSF and represent the mean $\pm$ s.e. mean. The number of samples is shown in brackets.

* significantly different from (a) and (c); the analysis of variance and a multiple range test was used. (Duncan, 1955).

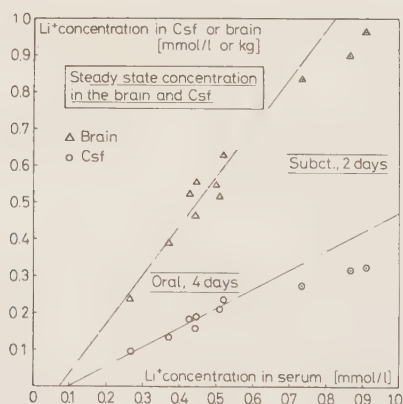


FIG. 7.0. Raw data from the feeding experiment (Table 7.1) were used to construct the regression lines. The first three sets of concentrations after 2 days of repeated subcutaneous injections (Fig. 7.3) are shown for comparison.

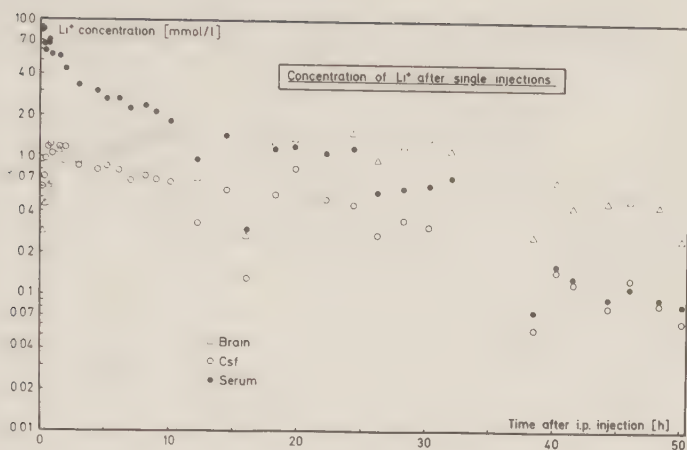


FIG. 7.1

Concentration in rats of Li^+ after single intraperitoneal injections of LiCl (5 mmol/kg body wt.). In all figures the Li^+ was measured in serum and CSF as mmol/l, and in whole brain as mmol/kg wet tissue.

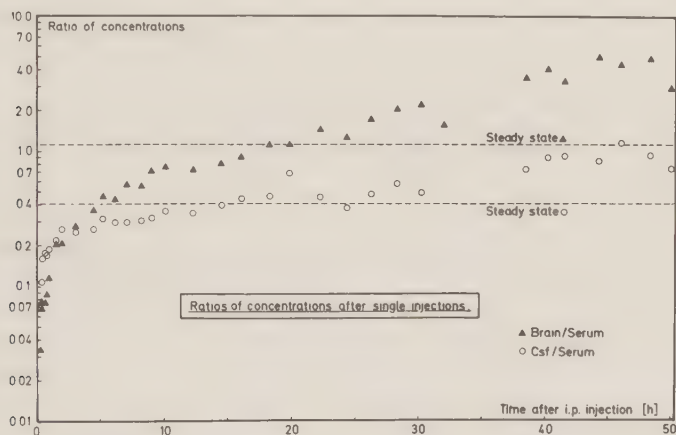


FIG. 7.2

Ratios of concentrations after single intraperitoneal injections. Concentrations of Li^+ (Fig. 7.1) replotted as Li^+ in CSF/serum and brain/serum, respectively. The broken lines represent the steady state values (Table 7.1).

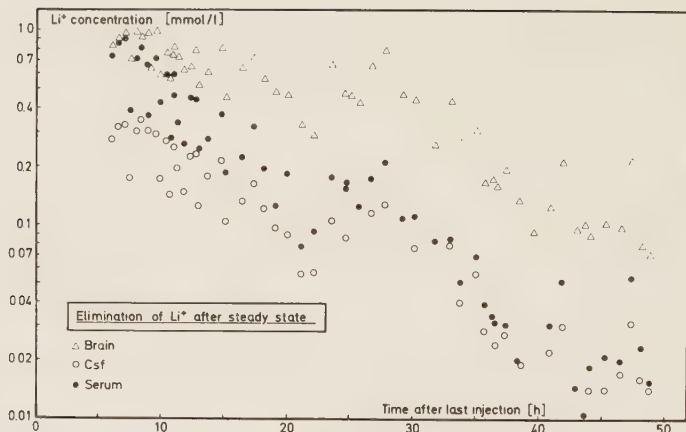


FIG. 7.3

Rats were given LiCl subcutaneously (0.9 mmol/kg body wt.) every 6 h for 2 days (approx. 8 half-lives). At the time indicated after the last injection, Li^+ was measured in serum, CSF and whole brain.

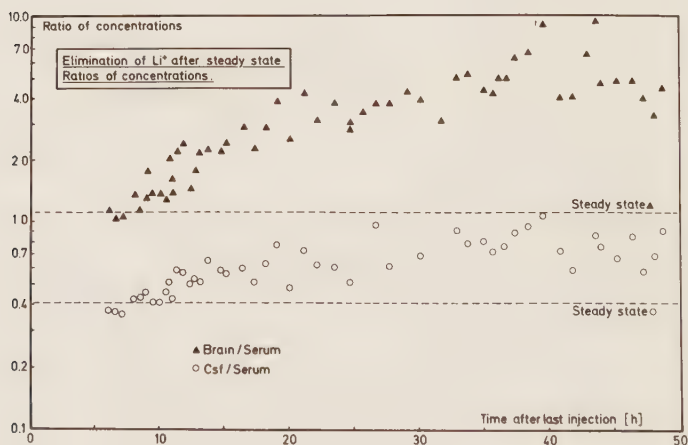


FIG. 7.4

Concentrations of Li^+ (Fig. 7.3) replotted as Li^+ in CSF/serum and brain/serum, respectively. The broken lines represent the steady state values (Table 7.1).

TABLE 7.2 STATISTICAL ANALYSIS OF RESULTS IN FIG. 7.3

Time after last injection:	Biological half-life (h)		
	mean	95%-confidence limits +	No. of samples
i) 6 h - 24 h			
Serum	5.35*	4.47 - 6.65	29
CSF	7.18*	5.83 - 9.35	27
Brain	12.73*	9.86 - 17.99	29
ii) 24 h - 48 h			
Serum	6.82 (n.s.)	5.43 - 9.18	28
CSF	7.57 (n.s.)	5.85 - 10.73	22
Brain	8.22 (n.s.)	6.88 - 10.21	28

* All three values were significantly different from each other.
(Overall variation: $P < 0.001$; individual differences: $P < 0.05$);
n.s. = not significant. The analysis of variance and a multiple
range test was used (Duncan, 1955).

+ The asymmetry around the means is due to the transformation from
a semilogarithmic plot.

to solutes so that most of the differences in the rate of penetration of solutes through the barriers could be explained by the more favourable area-to-volume relationship of the brain (Davson, 1967, p. 269). Therefore, it would seem likely that initially the concentration of Li^+ in the extracellular fluid of the cerebral tissue is equal to, or higher than that of the CSF and that the delay in uptake is due to the neuronal and glial membranes, resulting in a concentration of Li^+ which appears to be low because it referred to total wet weight in the present experiments. The delay due to neuronal and glial membranes was investigated earlier. The net uptake of Li^+ into cerebral slices reached a steady state after approx. 1 h (Chapter Three, Fig. 3.1 and 3.2). This alone can hardly explain the difference between the concentration maxima of Li^+ in the CSF and the cerebral tissue in vivo of several hours. However, the extracellular fluid of the slices was in steady state with respect to Li^+ after 1 - 2 min (calculations of Chapter Three), whereas the concentration of Li^+ in the extracellular fluid of the intact brain increased much more slowly and never reached a steady state after single injections. This explanation may make the two findings compatible. Under experimental circumstances comparable to the present, Bakay (1960) found that $^{24}\text{Na}^+$ appeared in the CSF before the cerebral tissue, whereas the activities of $^{24}\text{Na}^+$ in the CSF and brain were approx. simultaneous if constant plasma levels of $^{24}\text{Na}^+$ were maintained (Davson, 1967, p. 124 - 125).

Except during the first few hours after a single injection, the concentration of Li^+ was lower in the CSF than in both the cerebral tissue and serum. However, it is also obvious from a plot of the ratios (Fig. 7.2) that one cannot draw meaningful conclusions about the steady state from experiments with single doses of Li^+ (Baker & Winokur, 1966; Smith & Balagura, 1972).

The ratios at steady state in the present experiments were

reproducible for each of the two different routes of administration (Fig. 7.0). However, from the ratios of the concentration in the CSF and serum during elimination after steady state (Fig. 7.4) one can infer that, although the influence of a single dose is less in this situation, the magnitude of the dose and the interval after the last dose would influence the ratios, mainly, because of the fluctuations in the serum levels. This is the most likely explanation of the different ratios reported in the literature even under steady state conditions (Schou et al., 1954; Gershon & Yuwiler, 1960; Paul et al., 1973; Watanabe et al., 1973). Consequently, the conditions under which the samples were taken should also be stated when Li^+ in the CSF is measured.

The ratio of Li^+ in the CSF and serum varies because of the factors mentioned above, but it is consistently less than one. Li^+ is not bound to the proteins in the serum so that the CSF does not represent simply an ultrafiltrate of the serum with respect to Li^+ . On the other hand, there was a linear relationship at steady state between the concentration in the serum and the CSF both in the present experiments (Fig. 7.0) and in patients treated with Li^+ (Paul et al., 1973; Watanabe et al., 1973). This is in contrast to the constant composition of the CSF with respect to K^+ , Ca^{2+} and Mg^{2+} , and is generally taken as indicating passive transfer (Davson, 1967, p. 140 - 148). Prockop & Marcus (1972), from their experiments on rabbit choroid plexus in vitro, and from ventriculo-cisternal perfusion in dogs, concluded that simple diffusion and bulk flow could account for most of the clearance of Li^+ from the CSF; similar results were found by Hesketh & Glen (1978). The passage of water into the CSF is very rapid (Davson, 1967, p. 76 - 77), whereas the entry of Li^+ is slow, as demonstrated in the present experiments. This difference alone in the rate of entry of water and lithium would be sufficient to explain the continuous removal of lithium by bulk flow of the CSF through the arachnoid villi back to the serum,

and hence the ratio.

The ependymal tissue offers very little resistance to inulin, Na^+ , K^+ , and other solutes (Rall et al., 1962; Davson & Pollay, 1963; Cserr, 1965; Davson, 1967, p. 178 - 181). Therefore, the extracellular fluid of the cerebral tissue can be considered in equilibrium with the CSF, having concentrations which are probably not much higher. This would mean that a concentration gradient exists between the cerebral cells and the surrounding fluid at steady state. The findings in vivo are thus in agreement with earlier findings in cerebral slices (Kjeldsen et al., 1973; Chapter Three, Four and Six).

Li^+ is probably eliminated from the cerebral tissue via the CSF. The concentration ratio between the brain and the CSF would favour this pathway and removal from the CSF by bulk flow would be independent of the concentration of Li^+ in the serum. Elimination through the blood-brain barrier directly to the serum would seem unlikely under normal circumstances. The kinetic findings in the present experiments are consistent with the former possibility. Firstly, the initial rate of elimination from the CSF was intermediate between that of the serum and the cerebral tissue (Table 7.2 i)). Secondly, the concentration ratio of Li^+ between the CSF and serum, and between the brain and CSF during the β -phase were both approx. twice the corresponding ratios at the steady state, as can be inferred from Fig. 7.4.

The ratio of Li^+ between the CSF and serum at steady state in the present experiments was 0.41. In long term treatment of patients with Li^+ , Paul et al., (1973) and Watanabe et al. (1973) found corresponding ratios of 0.30 and 0.25, respectively. Li^+ was measured in the CSF and serum of patients from 1 h to 72 h after a single dose of lithium carbonate (0.2 mmol/kg body wt.) by Lehmann et al., (1976). Taking into account the differences due to the dose and route of administration, the temporal relationship between Li^+ in the CSF

and the serum in the present experiments after single dose (Fig. 7.1) was very similar to that found by these authors. In view of such similarities between the measurements of Li^+ in patients and in rats, it would seem reasonable to try to draw conclusions about the concentrations in the cerebral tissue in patients under comparable conditions. In rats, after a large single dose of Li^+ , the steady state ratio between Li^+ in the cerebral tissue and serum is reached after approx. 20 h. A similar pattern would explain the frequent absence of clinical signs in patients after an acute overdosage of Li^+ , although the serum values of Li^+ are high. When Li^+ was discontinued after repeated doses the ratio of Li^+ between the brain and serum in the present experiments increased to more than 4 times the ratio at steady state. The latter finding might explain why the clinical signs of Li^+ intoxication may persist for several days after serum concentrations of Li^+ have fallen. However, it does not explain why some patients take weeks to recover. Also, the use of haemodialysis in the treatment of Li^+ intoxication modifies the kinetics very much, by increasing greatly the rate of elimination of Li^+ from the serum, after which it falls to low values. This would lead to a ratio near one between Li^+ in the CSF and serum during haemodialysis, and a ratio approximating that at steady state afterwards (Amdisen & Skjoldborg, 1969; Hansen & Amdisen, 1978, Fig. 5b). Therefore, it would appear that the concentration in the cerebral tissue is approximately that of the serum some hours after redistribution of Li^+ in the tissue following haemodialysis. In these circumstances, measurement of Li^+ in the CSF would not reflect the concentration of Li^+ in the brain more accurately than measurement in the serum.

SUMMARY

1. Addition of lithium carbonate (55 mmol Li^+ / kg dry wt.) to the diet of rats for 4 days resulted in ratios between Li^+ in the brain and serum, between the cerebrospinal fluid (CSF)

and serum, and between the brain and CSF of approx. 1, 0.4, and approx. 3, respectively. The relationships between the concentrations were linear.

2. After single intraperitoneal injections of lithium chloride (5 mmol/kg body wt.) the concentration of Li^+ in the CSF was greater than that of the brain for 2 h.

3. Repeated subcutaneous injections of lithium chloride (0.9 mmol/kg body wt.) resulted in steady state ratios corresponding to those observed when Li^+ was given in the diet. The rate of elimination from the CSF was intermediate between that of the serum and cerebral tissue until a new equilibrium was reached after approx. 24 h. At that time the ratios between Li^+ in the brain and serum, and in the CSF and serum were increased to approx. 5 and 0.8, respectively.

4. These results are consistent with passive transfer kinetics of Li^+ in the CSF and elimination of Li^+ from the cerebral tissue via the CSF.

5. The results may explain some of the phenomena observed in patients during intoxication with Li^+ .

GENERAL DISCUSSION, SUMMARY AND CONCLUSIONS

Kinetics of Li^+ in red cells: In Chapter One, Four and Six some of the literature on Li^+ and red cells was mentioned briefly. This discussion begins with a review of some important results by others on transport mechanisms for Li^+ in human red cells. The results and reviews in the present thesis deal almost exclusively with excitable tissue. However, it is felt justifiable to include such a review on red cells in this final chapter, because transport systems are often common to many types of cells, and because the results on human red cells link the kinetic experiments of the present thesis with data from patients.

The general interest in Li^+ of red cells seems to have been triggered mainly by the finding of Frazer et al. (1973) on rats of the predictive value of Li^+ in red cells with respect to Li^+ levels in the brain during rapid changes of Li^+ in the serum, and that of Mendels and Frazer (1973) on manic-depressive patients of a correlation of Li^+ in red cells with the clinical response to treatment. The easy availability of red cells has been a further incentive to the studies. However, later attempts at using Li^+ in red cells to predict the efficacy of Li^+ treatment have demonstrated various sources of error in such experiments and have generally been unsuccessful (see review by Lee and Paschalis, 1978).

The kinetic studies have revealed a mechanism of transport for Li^+ not described earlier, besides showing that Li^+ shares some of the transport mechanisms with K^+ and Na^+ . In two independent studies Haas et al. (1975) and Duhm et al. (1976) using human red cells demonstrated net uphill trans-

port of Li^+ across the red cell membrane. The direction of the Li^+ flux was determined by the electrochemical gradient of Na^+ . Duhm *et al.* called the mechanism a Na-dependent Li^+ countertransport system. It was shown to be insensitive to ouabain and independent of ATP, but was later found sensitive to phloretin (Pandey *et al.*, 1976). An uptake of low concentrations of Li^+ via the K^+ site of the Na-pump as well as leak diffusion were also shown to operate in red cells (Duhm & Becker, 1977a). Interindividual differences in Li^+ treated patients of the concentration ratio of Li^+ between red cells and plasma were shown to reflect the activity of the Na-dependent Li^+ countertransport system alone, and tests of its activity *in vitro* could predict the concentration ratio *in vivo* (Greil *et al.*, 1977; Greil & Eisenried, 1978). However, the Li^+ ratio and the Na-Li countertransport system were found to be at least as variable in healthy donors (interindividual differences were up to five times) as in patients (Duhm & Becker, 1977c) and thus there cannot be any simple relation between the new transport system and manic-depressive disease. In red cells from healthy volunteers Dunham & Senyk (1977) could show Li^+ extrusion through the Na-pump. However, it was a prerequisite that the intracellular Na^+ was reduced to about 1 mM as the affinity of Li^+ for the Na^+ site was approx. 10 times lower than that of Na^+ (K_m values 50 mM and 5 mM, respectively). Therefore, this mechanism cannot be of any significance *in vivo*. An ATP-ase activated by low concentrations of Ca^{2+} was first found in membranes from red cells (Dunham & Glynn, 1961) and since demonstrated in other types of tissue (Baker & Reuter, 1975, p. 20 - 23). No effect of even high concentrations of Li^+ has been demonstrated on this enzyme system in red cell membrane (Bond & Green, 1971; Choi & Taylor, 1976).

In conclusion, the research activity on Li^+ and red cells has resulted in the discovery of a new transport system which accounts for the concentration ratio of Li^+ between red cells

and plasma under physiological circumstances. Transport of Li^+ via the K^+ site of the Na-pump takes place, but is quantitatively less important at normal concentrations of K^+ in the plasma. Extrusion of Li^+ via the Na-site of the pump is only observed at extremely low concentrations of Na^+ in the cells. There is no effect of Li^+ on the Ca-pump of red cells. None of the findings described seem to have any close relationship to the clinical course of manic-depressive disease.

Kinetics of Li^+ in cerebral slices: The transport mechanisms for Li^+ demonstrated unequivocally in red cells have not been found in the present experiments with cerebral slices. The experiments of Chapter Four (Fig. 4.3) and the control experiments of Chapter Five (Table 5.10) did not show any interaction between Li^+ and Na^+ suggestive of a Na-Li countertransport. There were some indications of Li^+ transport via the K^+ site of the Na-pump in the presence of low concentrations of K^+ in the medium (Fig. 4.2), but this could not be reproduced in the studies of the initial uptake in Chapter Five (Table 5.1 & 5.2). These differences between red cells and cerebral slices might be due to differences in the transport mechanisms, but might also be a question of experimental techniques.

Red cells are relatively robust. They can be incubated in the presence of highly unphysiological media and subjected to various inhibitions of transport mechanisms apparently without unintentional effects; the slow penetration of cations through the intact membrane of the red cell is an advantage in the creation of ionic concentration gradients; at the same time the permeability of the membrane towards cations can be reversibly increased allowing the red cells to be 'loaded' with one particular cation.

The cerebral slice is less ideal in these respects. The penetration of cations is faster than in the red cell and permeability changes are usually not reversible. The pronounced

swelling caused by metabolic inhibitors makes the interpretation of their effects on specific transport processes very difficult. During recent years various aspects of the established methods used in kinetic experiments on cerebral slices have been examined, calling attention to possible errors in the interpretation of experiments using these methods (Cohen, 1974 a & b; Hillman et al., 1974; Sershen & Lajtha, 1974; Lund-Andersen, 1974; Lund-Andersen & Kjeldsen, 1976; Lund-Andersen, 1978). Chapter Two of this thesis has dealt with these and other fundamental methodological problems in a review of the literature and presentation of control experiments. The failure to find firm evidence of the transport processes observed in red cells in the present experiments is unlikely to be due to the statistical methods or to the measurement of cations. The reference of concentrations to incubated weight has not biased any results seriously because the penetration of Li^+ is slow compared to its free diffusion; also, it has been possible throughout to estimate the consequences of presenting the results in this way. The effects of isolation and incubation seem to have been reasonably well controlled except for the oxygenation of the slices; though comparable to the functional activity reported in most of the literature on cerebral slices, the condition of the slices in the present experiments was not equivalent to the functional activity obtainable with the cutting table used (Franck et al., 1968; Arnfred et al., 1970). This has undoubtedly had some influence on the efflux experiments in which long incubation periods were used, cf. Table 6.1, but is unlikely to explain the negative results of Chapter Five as the incubations there lasted one to four min.

In addition to the indisputable heterogeneity of the cerebral slice due to differences in functional activity between the cells, there is an anatomical heterogeneity. Unlike the uniform red cells, the cerebral slices consist of at least two main types of cells. These could not be distinguished in

their kinetics of uptake towards Li^+ in a compartmental analysis (Chapter Three). However, the uptake of Li^+ in cultured neuroblastoma and glioma cells, which have been used as models of neurons and glial cells, respectively, is very different (Richelson, 1977; Gorkin & Richelson, 1979).

Li^+ itself imposes limits on kinetic experiments using cerebral slices. Experience from enzyme and transport kinetics shows that a range of low concentrations of the substrate is insufficient to demonstrate saturation unless K_m values are very low. The experiments of Chapter Five and the review of the literature there indicate that high concentrations of Li^+ have effects that may invalidate kinetic experiments, especially if the saturable part of the transport is so small compared with passive diffusion that a correction for the latter is necessary.

The experiments on Ca^{2+} and Li^+ in Chapter Five did not show any convincing indications of saturation. The effects of low concentrations of Ca^{2+} in the medium on Li^+ in the slices presented in Chapter Four were found unlikely to be accounted for by the known mechanisms of transport of Ca^{2+} . One of the other mechanisms suggested, namely a 'leak' in the membrane caused by low concentrations of Ca^{2+} in the medium would not exhibit saturation kinetics. Thus only the failure to demonstrate saturation of the Li^+ uptake in the presence of low concentrations of K^+ is most likely to be due to the experimental circumstances. However, in spite of the shortcomings of the methods of Chapter Five, the almost identical transfer constants obtained with media of differing cation composition and with low concentrations of Li^+ (Chapter Three) make it unlikely that any saturable mechanisms are quantitatively important in the uptake of Li^+ in cerebral slices.

This underlines the importance of efflux studies. However, contrary to expectations, the transfer constant determined for efflux turned out to be equal to that for influx. The pos-

sible reasons for this are discussed in Chapter Six, but no firm conclusions could be drawn. The measurements were insufficient for the application of Ussing's flux ratio test for active transport (Koefoed-Johnsen & Ussing, 1960, eq 6) but it is not unreasonable to assume that the flux ratios are smaller than the activity ratios, thus suggesting exchange diffusion (l.c. p. 173). This would point to future experiments investigating efflux of Li^+ in the presence of a high enough gradient between Na^+ in the slices and the medium to enable demonstration of a Na-dependent Li^+ countertransport. The experiments of this thesis were carried out before the publication of the experiments by Haas et al. (1975) and Duhm et al. (1976) so that the failure to find evidence of their transport system in cerebral slices may be due to the fact that the experiments of this thesis have not been planned with this in mind. Experiments like those by Haas et al., Fig. 2, involving transfer of the preparation from low Na^+ to high Na^+ media might be used as an initial approach. Due to effects on the membrane potential (Hillman & McIlwain, 1961) and the K^+ of the slices (Pappius et al., 1958) Na^+ concentrations as low as those of Haas et al. (10 mM in the medium) must be avoided. Furthermore, one would have to add choline⁺ in the presence of the necessary low concentrations of Na^+ in order to make up for the change in osmolarity. This has been avoided so far because choline⁺ cannot be considered inert in transport studies (Schuberth et al., 1966). The 'active' extrusion of Li^+ by the above mechanism depends critically on the electrochemical potential difference for Na^+ on the two sides of the membrane. The experiments by Hillman et al., (1974) and those of the present thesis (Fig. 4.3) indicate a fairly passive treatment of Na^+ by cerebral slices. However, this may be a question of the functional activity of the slices. Thus the conditions of incubation would probably have to be improved in order to demonstrate Na-dependent Li^+ countertransport in cerebral slices. If the slow component (Fig. 6.1) does represent efflux from damaged cells the cor-

responding compartment should then diminish or disappear. The number of experiments on slices required for the construction of the efflux curves would be considerable.

The kinetics of Li^+ in vivo: The slow uptake of Li^+ in vivo (Chapter Seven) contrasts with that found in cerebral slices. This difference between whole animals and cerebral slices is also seen in the measurement of the uptake in the brain of $^{42}\text{K}^+$ and $^{24}\text{Na}^+$ injected intraperitoneally into rats (e.g. Davson, 1967, Fig. 52; Bakay, 1960) and in the corresponding uptake into cerebral slices (e.g. Hertz, 1973, Fig. IV.2). The difference must be due first and foremost to the blood-brain barrier, but as was discussed in Chapter Seven, the uptake in the brain is delayed also due to the method of administration. Paradoxically, it would seem that the uptake of Li^+ into the cerebrospinal fluid is a more accurate reflection of the passage of Li^+ across the blood-brain barrier.

The concentration ratio of Li^+ between the cerebral tissue and cerebrospinal fluid at steady state was approx. 3, which is about twice as high as the ratio between the slices and the incubation fluid at steady state. Such a difference between the concentration ratios in vivo and in vitro is also observed for K^+ ; it is reasonable to regard this as a reflection of the functional state of cerebral slices compared with the living brain as both the K^+ and the Li^+ concentration in slices are dependent on metabolism. The concentration ratio of Li^+ between the serum and the cerebrospinal fluid observed at steady state in the present experiments was explained in Chapter Seven as being the result mainly of the bulk flow of the cerebrospinal fluid to the serum. There is no contradiction in postulating that this mechanism operates and that a concentration gradient is maintained between the cerebral tissue and the surrounding fluid by mechanisms requiring energy. The continuous flow of an extracellular fluid of relatively constant composition would not differ in principle

from a well stirred incubation medium and, as has been discussed earlier, the dependence of a concentration ratio on metabolism may be very indirect and not indicative of active transport.

The elimination of Li^+ at steady state from the cerebral tissue of intact animals was found likely to take place via the cerebrospinal fluid. This would correspond to the efflux of Li^+ from cerebral slices to the medium as there are no significant barriers between the extracellular fluid surrounding the cerebral cells and the cerebrospinal fluid. However, the efflux in vivo is not unidirectional, and recapture of Li^+ from the cerebrospinal fluid is probably sufficient to explain the slow initial elimination of Li^+ from the brain. There seems to be no particular affinity of cerebral tissue for Li^+ as shown by the evidence of a β -phase in the elimination in vivo.

The effects of changes in the cation composition of the medium on Li^+ in cerebral slices may be of little clinical relevance. However, Duhm & Becker (1977b, Fig.1) showed that a low K^+ in the serum increased the concentration ratio of Li^+ between red cells and serum and suggested that tissue Li^+ might increase without any changes in serum Li^+ in such a situation if the same mechanism operated in other cells. The nearest parallel in vivo to the experiments of Chapter Four would be ventriculo-cisternal perfusion with artificial cerebrospinal fluid of different cation composition. As shown by Prockop & Marcus (1972), there is no concentrative uptake of Li^+ in choroid plexus, which should make the interpretations of perfusion experiments relatively straightforward.

Summary and conclusions (results of relevant chapter indicated by Roman numerals): In living rats the concentration ratio of Li^+ between the cerebral tissue and the cerebrospinal fluid at steady state was approx. 3 (VII). In rat cerebral slices incubated in a Krebs-Ringer medium containing Li^+ 0.5 - 1.5 mM, the corresponding ratio was approx. 1.5 (IV) probably

due to a lower functional activity caused by the isolation and incubation (II). The ratio in cerebral slices was found to depend on the presence of glucose and oxygen (II & IV), on the concentration of K^+ , Ca^{2+} and, though less pronounced, of Mg^{2+} in the medium within fairly narrow limits, whereas concentrations of Na^+ and of Li^+ corresponding to those seen in the serum of patients had no significant effect on the ratio (IV). The rate of uptake of Li^+ was also found to depend on the oxygenation of the slices (III), but there was no evidence of a saturable uptake, neither within 'clinical' concentrations of Li^+ in the medium nor at concentrations up to 30 mM (V). The interaction of Li^+ with K^+ or Ca^{2+} observed at steady state was not apparent in the kinetics of the uptake (V). The experimental variation would obscure small effects and high concentrations of Li^+ do in themselves bias the results, but the findings (V) make it unlikely that saturable mechanisms are quantitatively important in the uptake of Li^+ in cerebral slices. The rate constants for influx (III) and efflux (VI) of Li^+ in cerebral slices were not compatible with each other or the concentration ratio of Li^+ between the tissue and the medium (III-IV & VI), either because the intracellular activity of Li^+ was different from its concentration, or because the experimental situations were not comparable. The concentration ratio suggests extrusion of Li^+ from the slices, but the data do not permit application of established criteria for active transport. The compartmental analysis of the net uptake showed only one component (III) whereas there were two in the efflux (VI). The slow component in the efflux was interpreted as being due to cell damage during efflux as suggested by the concentrations of K^+ of the slices (VI). The uptake of Li^+ in intact animals was much slower than that in cerebral slices (III & VII); this must be due mainly to the blood-brain barrier, but the method of administration also contributed to the difference. After 24 h the rate constants of elimination from the cerebral tissue, cerebrospinal fluid

and serum of the rats were not significantly different (VIII). This evidence of a β -phase in the elimination of Li^+ from the intact brain suggests that there is no particular affinity of cerebral tissue for Li^+ in vivo, nor was there any evidence of this in vitro (II). The interactions of Ca^{2+} and K^+ with Li^+ (IV) were not satisfactorily explained by the kinetic experiments (V), but the importance of Ca^{2+} for membrane permeability suggests leak diffusion, and evidence from other preparations of excitable tissue suggests a weak effect of Li^+ on a K^+ site of the Na-pump. The latter mechanism has also been observed in red cells, whereas the Na- Li^+ countertransport demonstrated in these cells was not seen in the present experiments (IV & V). Experiments are suggested which would be optimal for examining whether this transport system is present in cerebral tissue.

Kapitel I. Afhandlingens grundlag og disposition: Lithium-ionens (lithiums) fysisk-kemiske lighed med de biologisk vigtige kationer kalium, natrium, kalcium og magnesium og dens interaktion med cellemekanismer, som aktiveres og hæmmes af disse kationer, gør det sandsynligt, at lithium konkurrerer med disse ioner om cellulære transportmekanismer og måske udøver nogle af sine virkninger herved. Sådanne interaktioner skulle kunne iagttages i kinetiske undersøgelser. Da hjernen efter al sandsynlighed er effektororgan for lithiums antipsykotiske virkning, er det en naturlig forskningsopgave at undersøge lithiums kinetik og andre kationers indflydelse herpå i hjernevæv. Formålet med nærværende arbejde har været at undersøge lithiums kinetik i det strukturelt organiserede rottehjernevæv in vitro uden andre regulatoriske mekanismer end neuron- og gliacellemembranen, og i den intakte rottehjerne efter lithiumindgift in vivo. Bortset fra en velbegrundet undtagelse (kapitel 5) er der i forsøgene anvendt lithiumkoncentrationer sammenlignelige med serumværdierne hos patienter i lithiumbehandling; de øvrige kationer er undersøgt i et koncentrationsområde, som ikke afviger væsentligt fra det, som ses hos patienter under normale og patologiske omstændigheder. Til forsøgene in vitro er valgt snitpræparat af hjernebark ("hjerneslice"). Præparatets egenskaber og fordele ved transportstudier samt inkubationsmediets sammensætning gennemgås. Der foreligger kun få tidligere undersøgelser af lithiums kinetik i hjerneslices og kun et enkelt, som har haft dette som egentligt formål. Interaktion mellem lithium og kalcium, lithium og magnesium samt influx og efflux af lithium synes ikke tidligere at være undersøgt. In vivo er cerebrospinalvæsken

(CSF) den af de tilgængelige ekstracellulære væsker, som i almindelighed bedst afspejler farmakas indtrængen i hjernen. Den må ligeledes være det nærmeste anatomiske korrelat til inkubationsvæsken for hjerneslices. I undersøgelser på patienter i lithiumbehandling er der vist høj korrelation mellem koncentrationer af lithium i serum og i CSF ved steady state. Hos rotter er det vist, at når serumkoncentrationen af lithium ændres hurtigt, korrelerer den dårligt med koncentrationen i hjernen. Formålet med nærværende forsøg in vivo hos rotter har været dels ved en direkte sammenligning mellem koncentrationerne af lithium i serum, CSF og hjerne hos rotter at vurdere, om CSF-koncentrationen bedre end serumkoncentrationen kunne forudsige hjernekoncentrationen under optagelse og elimination, dels at sammenligne fundene med andres patientdata og egne data fra hjerneslices.

Kapitel II: Vurdering af resultater som er opnået ved hjælp af hjerneslices: I dette kapitel har forfatteren ved hjælp af litteraturstudier og egne forsøg belyst mulige fejlkilder ved præparation og inkubation af hjerneslices, måling af ekstracellulærrum i væv og ved statistiske metoder. Hjerneslices har trods optimale isolations- og inkubationsomstændigheder nedsat funktionel aktivitet i forhold til den levende hjerne, og præparatets funktionstid er begrænset til få timer. Som velkendte indikatorer for hjernevævets funktionelle aktivitet er i forsøg med hjerneslices brugt vægtforskellen mellem frisk og inkuberet væv som udtryk for den patologiske væskeoptagelse ("swelling") under forsøget, og kaliumkoncentrationen som udtryk for vævets evne til at arbejde mod en koncentrationsgradient. Det er tidligere vist, at tiden fra forsøgsdyrets død til inkubationen af hjerneslices er kritisk for disses funktionelle aktivitet. Når dette ikke har kunnet vises i rutineforsøg i nærværende arbejde, beror det formentlig dels på relativt små tidsforskelle ved præparationen under disse forsøg, dels at opbevaringen af

vævet forud for inkubationen har været optimal. Litteraturstudier gør det klart, at den uundgåelige afskæring af vævet anretter betydelige skader også på cellelag, der ligger dybere end selve skærezonen. Årsager hertil diskuteres. En maskinel, skæremetode, som hos andre forfattere har givet slices med høj funktionel aktivitet, er anvendt i nærværende forsøg, ligesom der kun er brugt slices med een snitflade. Da ilt og næringssubstrat må diffundere fra inkubationsmediet til de inderste cellelag er der en kritisk grænseværdi for tykkelsen af slices. Praktisk erfaring viser, at denne grænseværdi med fordel kan øges i forhold til Warburg's oprindelige udregninger. Årsager til dette diskuteres.

Inkubationsmediets sammensætning kan utilsigtet ændres under forsøgene. Dette kan ske ved fordampning, pH-ændring og diffusion af substanser fra slices til mediet. Den relative fordampning var ubetydelig på grund af forsøgsopstillingen og mediets store volumen; pH-ændringer mellem forsøgene var minimale, og det er af andre vist, at selv ret store ændringer i mediets pH under inkubationen kun i ringe grad reflekteres i vævets pH eller i den funktionelle aktivitet; substanser i mediet stammende fra slices kan sædvanligvis betragtes som betydningsløse i forsøg som i nærværende arbejde, undtagen i tilfælde hvor mediet nominelt er helt fri for den pågældende substans, og hvor små ændringer af substansen i et lavt koncentrationsområde giver store ændringer af lithiums transport (kalium, kapitel 4). Iltforsyningen til vævet er afgørende for dettes funktionelle aktivitet. For at opnå en optimal iltforsyning har det vist sig nødvendigt, at hjer-neslices uophørligt holdes i bevægelse af iltboblerne under inkubationen. Forsøgsopstillingen i nærværende forsøg har ikke sikret dette fuldtud, og nedsat iltforsyning synes at udgøre en metodologisk fejl i nogle af forsøgene; således er varigheden af forsøgene i kapitel 6 sandsynligvis kritisk på grund af dette.

Det er ved hjælp af homogeniseret væv undersøgt, om der skulle være en særlig stor affinitet mellem lithium og hjernevæv, idet lithium ikke nødvendigvis befinder sig i fri vandig opløsning intracellulært. Forsøgene viste ingen særlig affinitet, men metoden var ikke følsom. Formalinfikserede slices er i nærværende forsøg anvendt som model på ikke-metaboliserende væv, der havde samme struktur som en u-fikseret hjerneslice. De viste sig imidlertid uegnede, da de havde høj affinitet for natrium og lithium. Årsagerne til dette diskuteres.

Ved måling af lave koncentrationer af lithium i væv med forskelligt ionindhold kan interferensfænomener, som er uden betydning ved rutinebestemmelse af serumværdier blive meget generende. Selv med atomabsorptions-spektrofotometri, som er anvendt ved alle lithiumbestemmelser i nærværende arbejde, ses interferens i form af ionisation af beslægtede elementer og uspecifik absorption, ligesom præparationen før målingerne kan give anledning til co-præcipitation og viskositetsforskelle mellem prøve og standard. Derfor er der udført genfindingsforsøg under alle omstændigheder, hvor man af praktiske grunde ikke har kunnet opnå overensstemmelse mellem standard og prøve. Disse har alle vist tilfredsstillende målenøjagtighed. Til målinger af kalium og natrium er der brugt et flammefotometer af meget enkel konstruktion, hvorfor det også har været nødvendigt at kontrollere disse målinger. Genfindingsforsøg af samme type som ved lithiummålingerne har af naturlige grunde ikke kunnet udføres, men kontroleksperimenter har vist, at der kan ses bort fra alvorlige systematiske fejl.

Bestemmelse af den intracellulære lithiumkoncentration ville principielt være ønskelig. Imidlertid viser en gennemgang af litteraturen, at der ikke findes pålidelige rutinemetoder til bestemmelse af ekstracellulærrummet. De hyppigt anvendte markører, f.eks. inulin og rørsukker - selv i højt rensed form, har vist sig at passere cellemembra-

nerne i betydelig - og tidsafhængig grad i hjerneslices og andre præparater af eksitabelt væv. Væske, som adhærer til slices efter endt inkubation, udgør 2-15% af vægten ifølge litteraturen. Størrelsen af denne fejlkilde afhænger af, hvordan man definerer og bestemmer den "adhærente væske". I udregninger er der i nærværende arbejde regnet med en størrelse på 10% af den inkuberede vægt. Formalinfikserede slices forsøgte anvendt som kontrolpræparater til bestemmelse af ekstracellulærrum og adhærent væske, men fandtes uegnede. Forfatteren har derfor i sit arbejde relateret målte mængder af lithium, kalium og natrium til inkuberet vægt. Denne måde at angive koncentrationer på giver ved forsøg under steady state betingelser anledning til en forudsigelig systematisk fejl, hvorimod man ved kinetiske forsøg i hvert enkelt tilfælde må undersøge konsekvenserne for fortolkningen af forsøgene, hvilket er gjort i de følgende kapitlers diskussioner.

Den statistiske bearbejdning af resultaterne har været kritisk for fortolkningen, dels på grund af vanskeligt kontrollerbare forsøgsomstændigheder, dels på grund af sammenstyknings af resultater fra mange enkeltforsøg. Forudsætningerne for brugen af de anvendte statistiske prøver er undersøgt. Metodevalget ved multiple sammenligninger i nærværende arbejde begrundes mere udførligt, da det subjektive element i et sådant valg mellem "konservative" og "liberale" metoder er særlig udtalt ved disse prøver.

Kapitlet afsluttes med en kort gennemgang af almene problemer i fortolkningen af forsøgsresultater. I princippet bør man ved eksperimentelle undersøgelser af et biologisk spørgsmål anvende systemer af forskellig kompleksitet.

Kapitel III. Optagelse af lithium i hjerneslices ved lave koncentrationer: Influx (den initiale optagelse i perioden 15 s - 5 min) var proportional med koncentrationen af lithium i mediet i området fra 0,5 - 2 mmol/l, og der var så-

ledes ingen tegn på mætning af optagelsen i dette "kliniske" koncentrationsområde. Halvtiden var 7,9 min, sammenlignet med en beregnet halvtid for fri diffusion ind i ekstracellulærrummet i hjerneslices på 17 - 21 s. Den målte halvtid for influx påvirkes i nedadgående retning af diffusionen gennem ekstracellulærrummet og i opadgående retning af tilbagediffusion fra cellerne. Valget af inkubationstid påvirker disse modsat rettede tendenser og repræsenterer et kompromis dikteret af de praktiske muligheder. En teoretisk korrekt forskydning og forkortning af inkubationstiden for initialoptagelsen (kapitel 5) ændrer ikke resultaterne signifikant. Det forhold, at nogle stoffers frie diffusion er så langsom og deres membrantransport så hurtig, at denne sidste er umulig at måle, gælder ikke for en hurtig diffunderende og langsomt penetrerende ion som lithium. Lithiumoptagelsen nåede steady state efter en time. Optagelsen kunne ikke skelnes fra 1.ste ordens kinetik med eet intracellulært kompartment, og der er således ikke påvist målelige forskelle af lithiums optagelse i de to hovedtyper af hjerneceller. Når iltforsyningen til vævet erstattedes med kvælstof gik optagelseshastigheden for lithium ned til ca. 1/3. Denne afhængighed af vævsmetabolisme kan ikke umiddelbart tolkes som tegn på aktiv transport.

Kapitel IV. Lithium i hjerneslices ved steady state. Dens interaktion med andre kationer: Forholdet mellem lithiumkoncentrationen i vævet og mediet (0,5 - 1,5 mmol/l) var lineært med en koncentrationsratio på ca. 1,5. Når ilten erstattedes med kvælstof, eller når der ikke var sat glukose til mediet, ændredes denne ratio til ca. 1. Når kalium i mediet sænkedes indenfor koncentrationsområdet 0-10 mmol/l steg lithiumkoncentrationen i vævet non-lineært. Når mediet var (nominelt) kaliumfrit, var koncentrationsratio ca. 50% højere end ved standard-kaliumkoncentrationen 5 mmol/l.

Lignende forhold er påvist i andre præparater af eksitabelt væv og er i første omgang fortolket som konkurrence mellem lithium og kalium om en carrier for kalium på natriumpumpen. Der var et negativt lineært forhold mellem kalciumpkoncentrationen i mediet (0 - 5 mmol/l) og lithiumkoncentrationen i slices. Ingen af de kendte transportmekanismer for kalium kan forklare disse resultater. Kalciumionens betydning for membranpermeabilitet peger på uspecifik permeabilitetsøgning, men på grundlag af andres forsøg med subcellulære fraktioner af hjernehomogenater kunne man også tænke sig konkurrence mellem Li^+ og Ca^{2+} om en subcellulær receptor. Når magnesiumkoncentrationen i mediet øgedes fra de sædvanlige 1,3 mmol/l til det dobbelte, steg vævskoncentrationen af lithium med 10%. Ændringer i mediets koncentration af natrium inden for området 120 og 170 mmol/l havde ingen signifikant virkning på koncentrationen af lithium i slices. I lighed med tidligere publicerede fund fandtes i nærværende arbejde en lavere kaliumkoncentration i slices, der var inkuberet i medier med lave lithiumkoncentrationer end i slices fra medier uden lithium, men forskellen var ikke statistisk signifikant. Den statistiske prøve er diskuteret i kapitel 2.

Kapitel V. Undersøgelse af den initiale optagelse af lithium i hjerneslices og dens afhængighed af kalium og kalcium:

Lithiumkoncentrationerne ved de tidligere optagelsesforsøg (kapitel 3) er terapeutisk relevante, men det snævre koncentrationsområde begrænser samtidig anvendeligheden af initialhastigheden til at karakterisere optagelsen, idet måtning af eventuelle transportmekanismer meget vel kunne tænkes at finde sted ved væsentligt højere koncentrationer. I et forsøg på at undersøge, om der skulle være mætningskinetik for lithium i hjernevæv, og om den tidligere iagttagne interaktion mellem lithium, kalium og kalcium (kapitel 4) skyldes inhibering af optagelsen, og i så fald af hvilken art, anvendtes 8-10 mediekoncentrationer af lithium fra 1

til ca. 30 mmol/l; kalium og kalcium i inkubationsmediet varieredes som i de tidligere forsøg fra 0 til henholdsvis 10 og 5 mmol/l. Lineweaver-Burk's lineære transformation forsøgt korrigeret med statistiske vægte for at fjerne virkningen af den skævhed i punktfordelingen, som denne metode medfører.

Forsøgsresultaterne tydede ikke på mætning eller inhibering. Da det imidlertid er velkendt, at der kan være både mætbare og passive komponenter i optagelsen, og da en eventuel mætningskinetik i så fald vil være vanskelig at påvise på grund af den eksperimentelle spredning, blev initialoptagelsen desuden bestemt med lithiumkoncentrationer i mediet fra 40 til 120 mmol/l, hvor en eventuel faciliteret eller aktiv transport må antages at være mættet. Korrektur for passivt flux med Akedo-Christensens metode viste heller ingen tegn på mætning eller inhibering, men den nødvendige forudsætning for korrektionen - nemlig, at lithium i så høje koncentrationer kun virker ved at mætte eventuelle transportmekanismer - synes ikke at være opfyldt. Udregning af hastighedskonstanter, resp. halvtider, for optagelsen viste ingen signifikant forskel i optagelsen trods de nævnte variationer i ionsammensætningen af mediet og var heller ikke signifikant forskellige fra optagelseshastigheden målt ved lave koncentrationer.

Man må heraf slutte at mætbare transportmekanismer i hvert fald ikke kan være kvantitativt vigtige for lithiums optagelse i hjerneslices.

Kapitel VI. Efflux af lave lithiumkoncentrationer fra hjerneslices: Efflux målt under udvaskning i lithiumfrit medium i 150 min efter 1 times inkubation med en lithiumkoncentration i mediet på 1,5 mmol/l. Efflux var under disse forsøgsomstændigheder foreneligt med 1. ordens kinetik med to intracellulære kompartmenter. Ved hjælp af residualværdimetoden fandtes halvtider for de to faser på henholdsvis

9,7 min og 44,5 min; størrelsen af de tilsvarende kompartmenter - bestemt ved lineær extrapolation til tiden 0 - var henholdsvis 68% og 14% af lithiumkoncentrationen i mediet. Udregningen af kompartmenterne kompliceres, når disse udgør et seriesystem, hvorigennem en opløst substans må passere, før den når inkubationsmediet, specielt hvis hastighederne ikke er meget forskellige. Størrelsen af kompartmenterne er derfor senere udregnet med Huxley's korrektion under forskellige antagelser om kompartmenternes lokalisation og størrelsen af ekstracellulærrummet. Dette ændrer kun den samlede størrelse af det intracellulære kompartment ubetydeligt. Den hurtige fase er fortolket som udtryk for membranpassage på grund af hastigheden og på grund af størrelsen af det tilsvarende kompartment. Den langsomme fase repræsenterer antagelig efflux fra beskadigede celler. Med den anvendte teknik var det ikke muligt at registrere lithiums diffusion fra ekstracellulærrummet, men en skønsmæssig beregning af ekstracellulærrummet (30 μ l per 100 mg u-inkuberet våd vægt) ligger nær op ad publicerede værdier, som er opnået med en pålidelig måleteknik. De målte hastighedskonstanter, resp. halvtider, for influx og efflux var ikke signifikant forskellige, hvilket enten kan skyldes, at lithium ikke udelukkende er i fri vandig opløsning intracellulært, eller at forsøgsomstændighederne ikke er sammenlignelige. Koncentrationsratio for lithium mellem væv og inkubationsmedium ved steady state (kapitel 4) er betydelig lavere end beregnet efter Nernst-ligningen, hvilket taler for aktiv fjernelse af lithium fra cellerne, men måleresultaterne (kapitel 3, 4 og 6) er utilstrækkelige til anvendelse af Ussings flux ratio prøve for aktiv transport. Den lave koncentration af kalium i vævet under efflux tyder på lav funktionel aktivitet i hjerneslices efter de nødvendigt lange inkubationstider, og dette har sandsynligvis påvirket måleresultaterne, jf. den langsomme fase.

Kapitel VII. Lithiums kinetik i hjerne, cerebrospinalvæske og serum hos rotter: Indgift af lithiumkarbonat (55 mmol Li^+ per kg tørvægt) i rotternes foder gav efter 4 dage, hvor der må antages at være opnået steady state, koncentrationsratioer for lithium mellem hjerne og serum på ca. 1, mellem serum og CSF på 0,4 og mellem hjerne og CSF på ca. 3. Forholdet mellem koncentrationerne var lineært. Efter enkeltinjektioner af lithiumklorid intraperitonealt (5 mmol per kg legemsvægt) var koncentrationen af lithium i CSF højere end koncentrationen i hjernen i de første 2 timer. Til opnåelse af en præcist defineret steady state med små udsving i serum-lithium anvendtes subkutane injektioner hver 6. time i 2 dage (0,9 mmol per kg legemsvægt). Dette gav 6-8 timer efter sidste injektion koncentrationsratioer for lithium mellem hjerne, serum og CSF, der var identiske med dem, der målttes efter oral indgift. Eliminationshastigheden i CSF var lavere end eliminationshastigheden i serum og højere end den tilsvarende hastighed i hjernevævet, indtil en ny ligevægt indtrådte efter ca. 24 timer, idet hastighedskonstanterne, resp. halvtiderne, efter dette tidspunkt ikke længere var signifikant forskellige, et udtryk for den såkaldte β -fase. I denne fase var koncentrationsratio for lithium mellem henholdsvis hjerne og serum, og CSF og serum, steget til ca. 5 og 0,8. Koncentrationsændringerne i CSF og serum efter enkeltinjektioner viser, at man ikke, som det i tidligere arbejder er gjort, ud fra enkeltdoser kan konkludere noget om koncentrationsratio for lithium mellem disse ekstracellulærvæsker ved steady state. Imidlertid varierer den nævnte koncentrationsratio også i løbet af døgnet under steady state forhold, således at de noget vekslende angivelser i litteraturen af steady state ratio hos patienter antagelig kan forklares ved forskellig dosering og prøvetagningstidspunkt. Trods døgnsvingningerne er den nævnte ra-

tio altid mindre end 1. Dette skyldes hverken proteinbinding af lithium i serum eller aktiv fjernelse af lithium fra CSF, idet den sidste mulighed er afkræftet af andre ved hjælp af perfusion af hjerneventrikler og inkubationsforsøg med plexus chorioideus. Derimod kan den lave ratio utvungent forklares ved lithiums langsomme passage fra serum til CSF som vist i nærværende forsøg, den af andre påviste hurtige vandtransport til CSF og endelig den videre cirkulation af CSF gennem villi arachnoidales tilbage til serum ("bulk flow"). Lithiums elimination fra hjernen foregår sandsynligvis via CSF. Denne formodning støttes af de påviste forskelligheder i den initiale elimination af lithium fra hjerne, CSF og serum, og af stigningen i koncentrationsratio for lithium mellem CSF og serum, og mellem hjerne og CSF under β -fasen. Lithiums langsomme optagelse i og fjernelse fra hjernen kan rimeligvis forklare, at der ikke er kliniske forgiftningstegn hos patienter det første stykke tid efter en akut overdosering, selv om serumkoncentrationen på dette tidspunkt er høj, og omvendt, at der ses toksiske symptomer i flere dage efter, at serumkoncentrationerne er faldet. At toksiske symptomer hos nogle patienter først svinder flere uger efter, at lithium ikke længere har kunnet måles i serum, kan imidlertid ikke forklares af tilstedeværende lithium i hjernen, især da påvisningen af en β -fase i nærværende forsøg tyder mod en særlig affinitet mellem lithium og hjernevæv. Hvad angår spørgsmålet om forudsigelse af lithiumkoncentrationen i hjernen ved hjælp af de tilgængelige ekstracellulærvæsker, må man ud fra nærværende forsøg konkludere, at CSF-værdier ikke i denne henseende indebærer fordele fremfor serum-lithium. Denne konklusion støttes af andres nyligt publicerede patientdata fra hæmodialyse efter lithiumforgiftning. En sammenligning af funktione in vivo og in vitro er foretaget i det afsluttende kapitel.

Kapitel VIII. Afsluttende diskussion: Der har i de seneste år været en livlig udforskning af lithiums kinetik i røde blodlegemer. Resultater herfra er inddraget i diskussionen, da de meget vel kunne være relevante for eksitabelt væv, og da de er et bindeled mellem in vitro forsøgene i nærværende arbejde og patientdata. Med humane røde blodlegemer er det vist, at 3 transportmekanismer her er af betydning, nemlig passiv diffusion, natriumpumpens kaliumcarrier og et natrium-lithium modtransportsystem, som to forskergrupper har påvist uafhængigt af hinanden. De to første mekanismer vil normalt være uden større kvantitativ betydning, dog ville man under patologisk lave kaliumværdier i serum kunne se forhøjede lithiumkoncentrationer i røde blodlegemer, svarende til fundene i hjerneslices (kapitel 4), og det er ikke utænkeligt, at lignende forhold kunne gøre sig gældende i hjernevæv in vivo. Det er utvetydigt vist, at individuelle forskelligheder i koncentrationsratio for lithium mellem røde blodlegemer og serum hos frivillige forsøgspersoner og patienter i lithiumbehandling alene skyldes forskelle på aktiviteten i natrium-lithium modtransportsystemet. Imidlertid har man også fundet, at aktiviteten i det nævnte transportsystem og de individuelle forskelle i koncentrationsratio var lige så udtalte hos de frivillige forsøgspersoner som hos patienterne med manio-depressiv psykose. Der kan således ikke være nogen simpel relation mellem denne sygdom og det påviste transportsystem. Forsøg på at relatere koncentrationsratio for lithium mellem røde blodlegemer og serum til en lithiumbehandlings effektivitet har ligeledes været skuffende.

Bortset fra interaktionen mellem lithium og kalium (kapitel 4), der ikke kunne reproducere i de kinetiske forsøg (kapitel 5), er der kun få lighedspunkter mellem de tidligere nævnte fund i røde blodlegemer og nærværende fund i hjerneslices. Dette kunne selvsagt skyldes de åbenbare forskelligheder i celletyper, men kunne også være et spørgs-

mål om eksperimentel teknik. De robuste røde blodlegemer tåler bedre end hjerneslices de undertiden uundgåelige u-fysiologiske forsøgsomstændigheder. Anvendelsen af forskellige transportgifte, der har været et vigtigt led i forsøgene med røde blodlegemer, giver i hjerneslices en udtalt væskeoptagelse, der gør det vanskeligt at overskue deres virkning på bestemte transportsystemer. De senere års undersøgelser sætter spørgsmålstegn ved en del af de metoder, der tidligere har været almindeligt anvendt i kinetiske forsøg med hjerneslices. Kontrolforsøgene i kapitel 2 har dog vist, at den eneste alvorlige metodefejl i nærværende arbejde er en utilstrækkelig iltning af slices. Dette har som nævnt nok påvirket resultaterne i forsøg, hvor der er brugt lange inkubationstider, medens de negative resultater i kapitel 5, hvor inkubationen i lithiumholdige medier kun varede fra 1-4 min, næppe kan forklares med dette. Ensartetheden af de røde blodlegemer kontrasterer mod den anatomiske heterogenitet i hjerneslices. Selv om de to celletyper ikke kunne skelnes fra hinanden i nærværende kinetiske forsøg, er der i cellekulturer af andre påvist udtalte forskelle i optagelsen af lithium.

Lithium begrænser også ved sine egne virkninger anvendeligheden af hjerneslices til kinetiske forsøg. På den ene side er det, som tidligere nævnt, nødvendigt at undersøge kinetikken i et bredt koncentrationsområde for at påvise mætning, på den anden side har høje lithiumkoncentrationer virkninger, som vanskeliggør en fortolkning af sådanne forsøg. Koncentrationer op til 30 mmol/l synes dog med rimelighed at kunne anvendes, idet de fundne transportkonstanter ikke var signifikant forskellige fra dem, der opnåedes med "kliniske" lithiumkoncentrationer. Samtidig tyder koncentrationsuafhængigheden, som tidligere nævnt (kapitel 5) på, at mætbare transportmekanismer ikke kan være kvantitativt vigtige for lithiums optagelse i hjerneslices og dermed formentlig hjernevæv i det hele taget.

Dette understreger betydningen af efflux-målingerne (kapitel 6). Disse resulterede imidlertid i en hastighedskonstant, som var højere, end man ville vente med den fundne koncentrationsratio for lithium mellem væv og medium (kapitel 4). De mulige årsager hertil er tidligere diskuteret (kapitel 6). Som nævnt der, var måleresultaterne utilstrækkelige til påvisning af aktiv transport. Ud fra koncentrationsratio er det imidlertid ikke urimeligt at antage, at ratio mellem influx og efflux er mindre end ratio for lithiums elektrokemiske aktivitet mellem yderside og inderside af cellemembranerne, hvilket iflg. Ussing er tegn på ionbytning. Dette bringer natrium-lithium modtransportssystemet fra de røde blodlegemer ind i billedet. Når der ikke (kapitel 4) fandtes tegn på dette transportsystems tilstedeværelse, kunne det hænge sammen med, at forsøgsbetingelserne for dets påvisning ikke var optimale. Der skitseres derfor forsøg, som med større sikkerhed ville kunne afgøre dette spørgsmål.

Sammenligning af lithiums optagelse i hjernevæv in vivo (kapitel 7) og i hjerneslices (kapitel 3 og 5) viser store forskelle i hastighed. Noget tilsvarende er af andre tidligere vist ved radioaktivt kalium og natrium. Dette må skyldes blod-hjerne-barrieren. Imidlertid er optagelsen i hjernen også stærkt forsinket i forhold til CSF, hvilket må skyldes tilstedeværelsen af neuron- og gliamembraner. Det er usandsynligt, at der skulle være så store forskelle på lithiums penetration af disse membraner in vivo og in vitro. Derimod kan forskellen utvungent forklares ved, at koncentrationen af lithium i ekstracellulærvæsken in vivo, der er nogenlunde som i CSF, stiger langsomt, hvorimod den i ekstracellulærvæsken i hjerneslices vil nå konstant koncentration i løbet af få min. Paradoxsalt nok synes således optagelsen af lithium i CSF bedre end koncentrationen i hjernevævet at afspejle det tidsmæssige forløb af lithiums passage over blod-hjerne-barrieren.

Lithiums koncentrationsratio mellem hjernevævet og CSF var ca. 2 gange så høj som mellem hjerneslices og inkubationsmediet; noget tilsvarende finder man for kaliums vedkommende, og det må være et udtryk for forskellen i hjernevævetts funktionelle aktivitet i de to forskellige situationer, da koncentrationsratio for både kalium og lithium er afhængig af vævetts metabolisme. Dette er alligevel foreneligt med passiv fjernelse af lithium fra hjernevævet via CSF, idet der i princippet ikke er forskel på en cirkulerende ekstracellulærvæske af nogenlunde konstant sammensætning og et inkubationsmedium i konstant bevægelse. Koncentrationsratios afhængighed af den funktionelle aktivitet må efter de kinetiske forsøg (kapitel 3 og 5) være indirekte og bero på det eksitable vævs elektriske potential.

Påvisningen af en β -fase i lithiums elimination fra hjernevævet in vivo tyder ikke på nogen særlig affinitet mellem hjernevæv og lithium. Da der ikke er nogen diffusionsbarriere af betydning mellem ekstracellulærvæsken omkring hjernevævet og CSF, kunne det måske umiddelbart undre, at der er så stor forskel i eliminationshastigheden in vitro og in vivo. Imidlertid var efflux fra hjerneslices unidirektionel, medens lithiumkoncentrationen i CSF initialt var i steady state i forhold til hjernevævet og ændrede sig langsommere end serumkoncentrationen.

Det vil af ovenstående fremgå, at sammenligningen mellem hjerneslices og intakte hjerner vanskeliggøres af, at forsøgsbetingelserne er så forskellige. Det ville være muligt at undersøge lithiumionens bevægelser over neuron- og gliacellemembranen in vivo ved hjælp af hjerneventrikel-perfusion med kunstig CSF af forskellig ionsammensætning. Teknikken er velkendt. Een af dens fejkilder er tilstedeværelsen af plexus chorioideus. Fra andres forsøg vides det imidlertid, at lithium ikke koncentrerer i isolerede plexus, således at det skulle være enkelt at korrigere for denne fejlkilde. Et andet problem ville være den ringe føl-

somhed af lithiummålingerne (kapitel 2); det ville derfor være nødvendigt at anvende større dyr end rotter til hjerneventrikelperfusion, hvilket straks ville reducere fordelene ved sammenligning med resultater fra forsøg med rottehjerneslices. Desuden må man overveje berettigelsen af at anvende en så forholdsvis ressourcekrævende teknik i de videre undersøgelser på baggrund af den passive diffusionskinetik, der er påvist for lithium i nærværende arbejde med hjerneslices.

R E F E R E N C E S

Numbers in brackets refer to page number of this thesis. (A hyphen between two page numbers indicates that the reference is essential to that part of the thesis).

- Akedo, H. & Christensen, H.N.: Nature of insulin action on amino acid uptake by the isolated diaphragm. J. Biol. Chem. 1962, 237:118 - 122 (164)
- Amdisen, A.: Serum lithium determinations for clinical use. Scand. J. Clin. Lab. Invest. 1967, 20: 104 - 108 (59)
- Amdisen, A.: The estimation of lithium in urine. In Johnson, F.N. ed.: Lithium research and therapy. Academic Press, London, New York & San Francisco 1975, p.p. 181 - 195 (61)
- Amdisen, A. & Skjoldborg, H.: Haemodialysis for lithium poisoning. Lancet II, 1969: 213 (193)
- Ames, A., Tsukada, Y. & Nesbett, F.B.: Intracellular Cl^- , Na^+ , K^+ , Ca^{2+} , Mg^{2+} , and P in nervous tissue; response to glutamate and to changes in extracellular calcium. J. Neurochem. 1967, 14: 145 - 159 (27,134)
- Andersen, B. & Jørgensen, O.S.: Enzymkinetik I (2. udg.) & II. Kemisk Laboratorium IV, København 1969 & 1970 (24)
- Araki, T., Ito, M., Kostyuk, P.G., Oscarsson, O. & Oshima, T.: The effects of alkaline cations on the responses of cat spinal motoneurons, and their removal from the cells. Proc. Roy. Soc. Series B 1965, 162: 319 - 332 (178)
- Arnfred, T., Hertz, L., Lolle, L. & Lund-Andersen, H.: An improved holder for transfer of brain slices during in vitro incubation. Exp. Brain Res. 1970, 11: 373-375 (35,39,40,198)
- Askelöf, P., Korsfeldt, M., & Mannervik, B.: Error structure of enzyme kinetic experiments. Implications for weighting in regression analysis of experimental data. Eur. J. Biochem. 1976, 69: 61 - 67 (155)

- Baastrup, P.C., Poulsen, J.C., Schou, M., Thomsen, K. & Am-
disen, A.: Prophylactic lithium: Double blind discontin-
uation in manic-depressive and recurrent-depressive dis-
orders. Lancet II, 1970: 326 - 330 (17)
- Bachelard, H.S, Campbell, W.J. & McIlwain, H.: The sodium
and other ions of mammalian cerebral tissues, maintained
and electrically stimulated in vitro. Biochem. J. 1962,
84: 225 - 232 (29,30,36,41)
- Bailey, N.T.J.: Statistical methods in biology. English Uni-
versities Press, London 1959, p. 51 (53,88,100)
- Bakay, L.: Studies in sodium exchange. Neurology 1960, 10:
564 - 571 (190,201)
- Baker, M.A. & Winokur, G.: Cerebrospinal fluid lithium in
manic illness. Br. J. Psychiat. 1966, 112:163 - 165 (190)
- Baker, P.F.: Phosphorus metabolism of intact crab nerve and
its relation to the active transport of ions. J. Physiol.
(Lond.) 1965, 180: 383 - 423 (131,178)
- Baker, P.F. & Reuter, H.: Calcium movement in excitable
cells. Series: Pergamon studies in the life sciences. Per-
gamon Press, Oxford, New York, Toronto, Sydney & Braun-
schweig 1975 (132,133,196)
- Bartlett, M.S.: Properties of sufficiency and statistical
tests. Proc. Roy. Soc. Series A 1937, 160:268-282 (84,101)
- Bassir, O.: Molecular inhomogeneity as a source of error in
inulin clearance studies. J.Physiol. (Lond.) 1956, 131:
586 - 591 (75)
- Beaugé, L.: The interaction of lithium ions with the sodium-
potassium pump in frog skeletal muscle. J.Physiol. (Lond.)
1975, 246: 397 - 420 (128,129,130)
- Beaugé, L.A. & Ortiz, O.: Further evidence for a potassium-
like action of lithium ions on sodium efflux in frog skel-
etal muscle. J.Physiol. (Lond.) 1972, 226:675 - 697

(128,129)

- Bianchi, C.P., Frazer, A. & Mendels, J.: Lithium alteration of sodium-potassium transport ratio in sodium loaded sciatic nerve. Fed.Proc. Fed.Am. Socs. Exp. Biol. 1977,36: 989 (126,128,129,130)
- Birch, N.J.: Metabolic effects of lithium. In Johnson, F.N. & Johnson, S. eds.: Lithium in medical practice. M.T.P. Press, Lancaster, U.K. 1978, p.p. 89 - 114 (135)
- Blijenberg, B.G. & Leijnse, B.: The determination of lithium in serum by atomic absorption spectroscopy and flame emission spectroscopy. Clin. Chim. Acta 1968, 19: 97 - 99 (64)
- Bliss, C.I.: Statistics in biology, volume I, McGraw-Hill, New York 1967 (88,112,171)
- Bond, G.H. & Green, J.W.: Effects of monovalent cations on the ($Mg^{2+} + Ca^{2+}$)-dependent ATPase of the red cell membrane. Biochim. Biophys. Acta 1971, 241: 393-398 (196)
- Bourke, R.S. & Tower, D.B.: Fluid compartmentation and electrolytes of cat cerebral cortex in vitro. I. Swelling and solute distribution in mature cerebral cortex. J. Neurochem. 1966a, 13: 1071 - 1097 (41)
- Bourke, R.S. & Tower, D.B.: Fluid compartmentation and electrolytes of cat cerebral cortex in vitro. II. Sodium, potassium and chloride of mature cerebral cortex. J. Neurochem. 1966b, 13: 1099 - 1117 (41,130,135)
- Brading, A.F.: Sodium/sodium exchange in the smooth muscle of the guinea-pig taenia coli. J. Physiol.(Lond.) 1975, 251: 79 - 105 (131)
- Brown, D.A., Stumpf, W.E. & Roth, L.J.: Location of radioactively labelled extracellular fluid indicators in nervous tissue by autoradiography. J. Cell. Sci. 1969,4: 265 - 288 (75)

- Bunney, W.E. & Murphy, D.L. eds. The neurobiology of lithium. Neurosci. Res. Prog. Bull. 1976, 14: 110 - 207(17)
- Cade, J.F.J.: Lithium salts in the treatment of psychotic excitement. Med. J. Aust. 1949, 2: 349 - 352 (17)
- Campbell, R.C.: Statistics for biologists, 2nd ed. Cambridge University Press 1974 (87,92)
- Carpenter, B.S., Samuel, D., Wassermann, I. & Yuwiler, A.: A study of lithium uptake and location in the brain using the nuclear track technique. J. Radioanal. Chem. 1977, 37: 523 - 528 (21)
- Choi, S.J. & Taylor, M.A.: Effect of lithium on Ca^{++} -A.T.P.-ase. Lancet II, 1976: 1080 (196)
- Christensen, H.N.: Biological transport, 2nd ed. W.A. Benjamin, London, Amsterdam, Ontario, Sydney & Tokyo 1975 (17,131,133,134,141,160)
- Christensen, H.N. & Liang, M.: Modes of uptake of benzylamine by the Erlich cell. J. Biol. Chem. 1966, 241: 5552 - 5556 (156,164)
- Christensen, S.: Accumulation of lithium in the particulate fraction of rat brain following its long-term administration. J. Neurochem. 1974, 23: 1299 - 1301 (125,129)
- Christian, G.D. & Feldman, F.J.: Atomic absorption spectroscopy; applications in agriculture, biology, and medicine. Wiley, New York, London, Sydney & Toronto 1970 (57)
- Cochran, W.G.: The combination of estimates from different experiments. Biometrics 1954, 10: 101 - 129 (84-86)
- Cohen, S.R.: The estimation of extracellular space of brain tissue in vitro. In Marks, N. & Rodnight, R. eds. Research methods in neurochemistry, volume I, Plenum Press, New York & London 1972 p.p. 179 - 219 (72-76)
- Cohen, S.R.: The rate of respiration of mouse brain tissue in physiological and non-physiological media as a

- function of temperature. Comp. Biochem. Physiol. 1973, 45A: 1031 - 1045 (38)
- Cohen, S.R.: The contribution of adherent films of liquid on the cut surfaces of mouse brain slices to tissue water and "extracellular" marker spaces. Exp. Brain Res. 1974a, 20: 421 - 434 (32,78-80,198)
- Cohen, S.R.: The dependence of water content and "extracellular" marker spaces of incubated mouse brain slices on thickness; alterations produced by slicing, and fluid spaces in intact and altered tissue. Exp. Brain Res. 1974b, 20: 435 - 457 (32-33,173,198)
- Cohen, S.R.: A comparison of the rate equations, kinetic parameters, and activation energies for the initial uptake of l-lysine, l-valine, γ -aminobutyric acid, and α -aminobutyric acid by mouse brain slices. J. Membrane Biol. 1975, 22: 53 - 72 (83,156)
- Cohen, S.R., Blasberg, R., Levi, G. & Lajtha, A.: Compartmentation of the inulin space in mouse brain slices. J. Neurochem. 1968, 15: 707 - 720 (71,75)
- Concise Oxford Dictionary 5th ed. Oxford University Press, 1964 (92)
- Coombs, H.I.: The estimation of lithium in serum. Br. J. Psychiat. 1971, 118: 225 - 226 (60)
- Cserr, H.: Potassium exchange between cerebrospinal fluid, plasma and brain. Am. J. Physiol. 1965, 209: 1219 - 1226 (192)
- Cummins, J.T. & McIlwain, H.: Electrical pulses and the potassium and other ions of isolated cerebral tissues. Biochem. J. 1961, 79: 330 - 341 (62)
- Dainty, J. & House, C.R.: 'Unstirred layers' in frog skin. J. Physiol. (Lond.) 1966, 182: 66 - 78 (82)
- Dale, H.: Accident and opportunism in medical research. Br.

- med. J. 1948, 2: 451 - 455 (18)
- Davenport, V.D.: Distribution of parenterally administered lithium in plasma, brain and muscle of rats. Am. J. Psychol. 1950, 163: 633 - 641 (19)
- Davies, M.: Functions of biological membranes. Series: Outline studies in biology. Chapman & Hall, London 1973 (24)
- Davson, H.: Physiology of the cerebrospinal fluid. Churchill, London 1967 (190-192,201)
- Davson, H. & Pollay, M.: The turnover of ^{24}Na in the cerebrospinal fluid and its bearing on the blood-brain barrier. J. Physiol. (Lond.) 1963, 167: 247 - 255 (192)
- Dawes, E.A.: Quantitative problems in biochemistry. 5th ed. Churchill Livingstone, Edinburgh & London 1972 (24)
- Dawson, J. & Bone, A.: Water uptake by, and sodium and potassium content of, brain slices. J. Neurochem. 1965, 12: 167 - 180 (27)
- Dickens, F. & Greville, G.D.: The metabolism of normal and tumor tissue. XIII. neutral salt effects. Biochem. J. 1935, 29: 1468 - 1483 (135)
- Documenta Geigy: Scientific tables 6th ed., J.R. Geigy, Basle 1962 (63,72)
- Dowd, J.E. & Riggs, D.S.: A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. J. Biol. Chem. 1965, 240: 863 - 869 (161)
- Duhm, J. & Becker, B.F.: Studies on the lithium transport across the red cell membrane.II. Characterization of ouabain-sensitive and ouabain-insensitive Li^+ transport. Effects of bicarbonate and dipyridamole. Pflügers Arch. 1977a, 367: 211 - 219 (196)
- Duhm, J. & Becker, B.F.: Studies on the lithium transport across the red cell membrane.III. Factors contributing to the intraindividual variability of the in vitro Li^+ distrib-

- ution across the human red cell membrane. Pflügers Arch. 1977b, 368: 203 - 208 (202)
- Duhm, J. & Becker, B.F.: Studies on the lithium transport across the red cell membrane. IV. Interindividual variations in the Na^+ -dependent Li^+ countertransport system of human erythrocytes. Pflügers Arch. 1977c, 370: 211-219 (196)
- Duhm, J., Eisenreid, F., Becker, B.F. & Greil, W.: Studies on the lithium transport across the red cell membrane. I. Li^+ uphill transport by the Na^+ -dependent Li^+ countertransport system of human erythrocytes. Pflügers Arch. 1976, 364: 147 - 155 (131,195,196,200)
- Duncan, D.B.: Multiple range and multiple F tests. Biometrics 1955, 11: 1 - 42 (54,90-92,113,120,162,186,189)
- Dunham, E.T. & Glynn, I.M.: Adenosinetriphosphatase activity and the active movements of alkali metal ions. J. Physiol. (Lond.) 1961, 156: 274 - 293 (196)
- Dunham, P.B. & Senyk, O.: Lithium efflux through the Na/K pump in human erythrocytes. Proc. Natl. Acad. Sci. USA 1977, 74: 3099 - 3103 (196)
- Dunn, O.J.: Multiple comparisons among means. J. Am. Statist. Assoc. 1961, 56: 52 - 64 (91)
- Dunnett, C.W.: New tables for multiple comparisons with a control. Biometrics 1964, 20: 482 - 491 (91)
- Ebadi, M.S., Simmons, V.J., Hendrickson, M.J. & Lacy, P.S.: Pharmacokinetics of lithium and its regional distribution in rat brain. Eur. J. Pharmac. 1974, 27: 324 - 329 (19)
- Elliott, K.A.C.: Swelling of brain slices and the permeability of brain cells to glucose. Proc. Soc. Exp. Biol. Med. 1946, 63: 234 - 236 (27)
- Elliott, K.A.C.: The use of brain slices. In Lajtha, A. ed. Handbook of Neurochemistry, volume 2, Plenum Press, New York & London 1969, p.p. 103 - 114 (30,33,35)

- Elliott, K.A.C. & Birmingham, M.K.: The effect of pH on the respiration of brain tissue; the pH of tissue slices. J. Biol. Chem. 1949, 177: 51 - 58 (38)
- Franck, G., Cornette, M. & Schoffeniels, E.: The cationic composition of incubated cerebral cortex slices. J. Neurochem. 1968, 15: 843-857 (27,30f,33,35f,38f,41,48,126,198)
- Frazer, A., Mendels, J., Secunda, S.K., Cochrane, C.M. & Bianchi, C.P.: The prediction of brain lithium concentrations from plasma or erythrocyte measures. J. Psychiat. Res. 1973, 10: 1 - 7 (19,20,181,195)
- Gardner, D.R. & Kerkut, G.A.: A comparison of the effects of sodium and lithium ions on action potentials from Helix aspersa neurones. Comp. Biochem. Physiol. 1968, 25: 33 - 48 (131,178)
- Geigy's Tables, see: Documenta Geigy
- Gershon, S. & Shopsin, B. eds.: Lithium; its role in psychiatric research and treatment. Plenum Press, New York & London 1973 (24)
- Gershon, S. & Yuwiler, A.: Lithium ion: a specific psychopharmacological approach to the treatment of mania. J. Neuropsychiat. 1960, 1: 229 - 241 (191)
- Gorkin, R.A. & Richelson, E.: Lithium ion accumulation by cultured glioma cells. Brain Res. 1979, 171: 365-368 (199)
- Greil, W. & Eisenried, F.: Lithium uptake by erythrocytes of lithium-treated patients: interindividual differences. In Johnson, F.N. & Johnson, S. eds. Lithium in medical practice. M.T.P. Press, Lancaster, U.K. 1978, p.p. 415 - 420 (127,196)
- Greil, W., Eisenried, F., Becker, B.F. & Duhm, J.: Inter-individual differences in the Na^+ -dependent Li^+ counter-transport system and in the Li^+ distribution ratio across the red cell membrane among Li^+ -treated patients. Psychopharmacology 1977, 53: 19 - 26 (196)

- Haas, M., Schooler, J. & Tosteson, D.C.: Coupling of lithium to sodium transport in human red cells. Nature (Lond.) 1975, 258: 425 - 427 (131,195,200)
- Hansen, H.E. & Amdisen, A.: Lithium intoxication. Q. Jl. Med. 1978, New Ser. 47: 123 - 144 (193)
- Harned, H.S. & Hildreth, C.L.: The differential diffusion coefficients of lithium and sodium chlorides in dilute aqueous solution at 25⁰. J. Am. Chem. Soc. 1951, 73: 650 - 652 (102)
- Harris, E.J. & Maizels, M.: The permeability of human erythrocytes to sodium. J. Physiol. (Lond.) 1951, 113: 506 - 524 (101,175,176)
- Haugaard, E.S., Frazer, A., Mendels, J. & Haugaard, N.: Metabolic and electrolyte changes produced by lithium ions in the isolated rat diaphragm. Biochem.Pharmac. 1975, 24: 1187 - 1191 (128,129)
- Heath, O.V.S.: Investigation by experiment. The Institute of Biology's studies in biology no. 23. Edward Arnold, London 1970 (93)
- Hertog, A. den & Ploeger, E.J.: Mechanism of action of lithium salts. Effects on excitable membranes. Psychiat. Neurol. Neurochir. (Amst.) 1973, 76: 529 - 535 (131)
- Hertz, L.: Potassium effects on ion transport in brain slices. J. Neurochem. 1968, 15: 1 - 16 (39)
- Hertz, L.: Ion effects on metabolism in the adult mammalian brain in vitro. Evidence of a potassium-induced stimulation of active uptake of KCl into neuroglial cells. Thesis, Odense, FADLs Forlag, København, Århus & Odense 1973 (27, 28,34,71,201)
- Hertz, L. & Schou, M.: Univalent cations and the respiration of brain-cortex slices. Biochem.J. 1962, 85: 93 - 104 (135,157,158)

- Hertz, L., Schousboe, A. & Weiss, G.B.: Estimation of ionic concentrations and intracellular pH in slices from different areas of rat brain. Acta Physiol. Scand. 1970, 79: 506 - 515 (31,38,76)
- Hesketh, J.E. & Glen, A.I.M.: Lithium transport from cerebrospinal fluid. Biochem. Pharmac. 1978, 27: 813 - 814 (191)
- Hesketh, J.E., Kinloch, N. & Reading, H.W.: The effects of lithium on ATPase activity in subcellular fractions from rat brain. J. Neurochem. 1977, 29: 883 - 894 (128,129)
- Hille, B.: Channels and ion transport in axons and synapses. In Bunney, W.E. & Murphy, D.L. eds. The neurobiology of lithium. Neurosci. Res. Prog. Bull 1976, 14: 161 - 164 (131)
- Hillman, H.: Certainty and uncertainty in biochemical techniques. Surrey University Press, Henley-on-Thames 1972 (92)
- Hillman, H.: Towards a classification of evidence in biological and medical research in respect of its validity. Acta Biotheor. 1976, 25: 153 - 162 (93)
- Hillman, H., Campbell, W.J. & McIlwain, H.: Membrane potentials in isolated and electrically stimulated mammalian cerebral cortex. J. Neurochem. 1963, 10: 325 - 339 (31)
- Hillman, H. & McIlwain, H.: Membrane potentials in mammalian cerebral tissues in vitro: dependence on ionic environment. J. Physiol. (Lond.) 1961, 157: 263-278 (30,38,135,157,178,200)
- Hillman, H., Stollery, S. & Joanny, P.: The mechanism of the exclusion of sodium ions and the concentration of potassium ions by guinea-pig cerebral cortex slices. J. Neurochem. 1974, 22: 573 - 579 (27,41,132,198,200)
- Hullin, R.P.: The effects of lithium on electrolyte balance and body fluids. In Johnson, F.N. ed. Lithium research and therapy. Academic Press, London, New York & San Francisco

- 1975, p.p. 359 - 379 (135)
- Huxley, A.F.: Appendix 2 of (Solomon, A.K.) Compartmental methods of kinetic analysis. In Comar, C.L. & Bronner, F. eds. Mineral metabolism 1A. Academic Press, New York 1960, p.p. 163 - 166 (108,167)
- Israel, Y., Kalant, H. & leBlanc, A.E.: Effects of lower alcohols on potassium transport and microsomal adenosine-triphosphatase activity of rat cerebral cortex. Biochem. J. 1966, 100: 27 - 33 (19,126,129)
- Joanny, P. & Hillman, H.: Further studies on the potassium and sodium concentrations of mammalian cerebral slices in vitro. J. Neurochem. 1964, 11: 413 - 422 (40,130,134)
- Joanny, P., Natali, J.-P., Hillman, H. & Corriol, J.: The uptake and efflux of l-phenylalanine and l-tyrosine from rat brain cerebral cortex slices. Biochem. J. 1973, 136: 77 - 82 (83)
- Johnson, F.N. ed.: Lithium research and therapy. Academic Press, London, New York & San Francisco 1975 (24)
- Johnson, F.N. & Johnson, S. eds. Lithium in medical practice. M.T.P. Press, Lancaster, U.K. 1978 (24)
- Keeseey, J.C., Wallgren, H. & McIlwain, H.: The sodium, potassium and chloride of cerebral tissues: maintenance, change on stimulation and subsequent recovery. Biochem. J. 1965, 95: 289 - 300 (31,38,40)
- Keynes, R.D. & Swan, R.C.: The permeability of frog muscle fibres to lithium ions. J. Physiol. (Lond.) 1959, 147: 626 - 638 (178)
- Kjeldsen, C.S., Lund-Andersen, H. & Hertz, L.: Effects of lithium ions in a pharmacological concentration on potassium and sodium ions in rat brain-cortex slices. Biochem. Soc. Trans. 1973, 1: 111 - 114 (19,22,125,126,127,128,129, 132,135,157,178,179,192)

- Kluge, H., Gröschel, W., Hartmann, W., Zahlten, W. & Wieczorek, V.: Adsorption von lithium- und kalziumionen an zerebrale membranfraktionen. Pharmakopsykiat. 1974, 7: 293 - 299 (134, 135)
- Koefoed-Johnsen, V. & Ussing, H.H.: Ion transport. In Comar, C.L. & Bronner, F. eds. Mineral metabolism I A. Academic Press, New York 1960, p.p. 169 - 203 (178, 200)
- Kotyk, A. & Janáček, K.: Cell membrane transport: principles and techniques, 2nd ed. Plenum Press, New York & London 1975 (102)
- Krebs, H.A.: Body size and tissue respiration. Biochem. Biophys. Acta 1950, 4: 249 - 269 (19, 135)
- Kudsk, F.N.: Farmakokinetik. FADLs Forlag, København, Århus & Odense 1976 (24)
- Leaf, A.: On the mechanism of fluid exchange of tissues in vitro. Biochem. J. 1956, 62: 241 - 248 (27, 113)
- Lee, C.R. & Paschalis, C.: Lithium metabolism as a guide to the selection of patients for lithium therapy. In Johnson, F.N. & Johnson, S. eds. Lithium in medical practice. M.T.P. Press, Lancaster, U.K. 1978, p.p. 365 - 379 (195)
- Lehmann, K.: Die kinetik von lithium in serum, leber und gehirn der ratte. Acta Biol. Med. Germ. 1975, 34: 1043 - 1047 (19)
- Lehmann, K., Scherber, A. & Graupner, K.: Konzentrationsverlauf von lithium im liquor und augenkammerwasser nach einmaliger oraler applikation beim menschen. Int. J. Clin. Pharmac. 1976, 13: 22 - 26 (192)
- Li, C.-L. & McIlwain, H.: Maintenance of resting membrane potentials in slices of mammalian cerebral cortex and other tissues in vitro. J. Physiol. (Lond.) 1957, 139: 178 - 190 (178)
- Lineweaver, H. & Burk, D.: The determination of enzyme dis-

- sociation constants. J. Am. Chem. Soc. 1934, 56: 658 - 666 (140,161)
- Lolley, R.N.: The calcium content of isolated cerebral tissues and their steady-state exchange of calcium. J. Neurochem. 1963, 10: 665 - 676 (132)
- Longmuir, I.S. & Bourke, A.: Application of Warburg's equation to tissue slices. Nature (Lond.) 1959, 184: 634 - 635 (34,35)
- Lund-Andersen, H.: Extracellular and intracellular distribution of inulin in rat brain cortex slices. Brain Res. 1974, 65: 239 - 254 (76,174,198)
- Lund-Andersen, H.: En undersøgelse af den cellulære glucose-transportmekanisme i hjernesnit (A study of the mechanism of cellular transport of glucose in cerebral slices). Thesis, København. Panum Instituttets Fællesafdeling, København 1978 (198)
- Lund-Andersen, H. & Hertz, L.: Effects of potassium and glutamate on swelling and on sodium and potassium content in brain-cortex slices from adult rats. Exp. Brain Res. 1970, 11: 199 - 212 (28,35,71)
- Lund-Andersen, H. & Kjeldsen, C.S.: Uptake of glucose analogues by rat brain cortex slices: a kinetic analysis based upon a model. J. Neurochem. 1976, 27: 361-368 (22,83,198)
- Marchbanks, R.M.: Ion transport and metabolism in brain. In Bittar, E.E. ed. Membranes and ion transport, volume II. Wiley-Interscience, London, New York, Sydney & Toronto 1970, p.p. 145 - 184 (24)
- Margoshes, M.: An introduction to flame photometry and a review of recent studies. In Nastuk, W.L. ed. Physical techniques in biological research, volume IV, Academic Press, New York & London 1962, p.p. 215 - 260 (57)
- McIlwain, H.: Glucose level, metabolism and response to

- electrical impulses in cerebral tissues from man and laboratory animals. Biochem. J. 1953, 55:618 - 624 (40)
- McIlwain, H. & Bachelard, H.S.: Biochemistry and the central nervous system, 4th ed. Churchill Livingstone, Edinburgh & London 1971 (18,19,28,34)
- McIlwain, H. & Rodnight, R.: Practical Neurochemistry. Churchill, London 1962 (19,30,34,35,37,48,53,60,71,77,98)
- McLennan, H.: The diffusion of potassium, sodium, sucrose and inulin in the extracellular spaces of mammalian tissues. Biochim.Biophys. Acta 1957, 24: 1 - 8 (102)
- Meis, L. de: Allosteric inhibition by alkali ions of the Ca^{2+} uptake and adenosine triphosphatase activity of skeletal muscle microsomes. J.Biol.Chem. 1971, 246: 4764 - 4773 (133)
- Mendels, J. & Frazer, A.: Intracellular lithium concentration and clinical response: towards a membrane theory of depression. J. Psychiat. Res. 1973, 10: 9 - 18 (195)
- Minami, S.: Versuche an überlebendem carcinomgewebe (atmung und glykolyse). Biochemische Zeitschrift 1923, 142:334 ff. Cited from Warburg, O. ed. Über den stoffwechsel der tumoren. Springer, Berlin 1926, p.p. 85 - 101 (29,34,35)
- Mukherjee, B.P., Bailey, P.T. & Pradhan, S.N.: Temporal and regional differences in brain concentrations of lithium in rats. Psychopharmac. 1976, 48: 119 - 121 (19,185)
- Møller, M., Møllgård, K., Lund-Andersen, H. & Hertz, L.: Concordance between morphological and biochemical estimates of fluid spaces in rat brain cortex slices. Exp. Brain Res. 1974, 22: 299 - 314 (27,33,46,80,173,174)
- Nicholls, J.G. & Wolfe, D.E.: Distribution of ^{14}C -labeled sucrose, inulin and dextran in extracellular spaces and in cells of the leech central nervous system. J.Neurophysiol. 1967, 30: 1574 - 1592 (75)

- Notari, R.E.: Biopharmaceutics and pharmacokinetics, an introduction, 2nd ed. Marcel Dekker, New York 1975 (24)
- Okamoto, K. & Quastel, J.H.: Water uptake and energy metabolism in brain slices from the rat. Biochem. J. 1970, 120: 25 - 36 (27,130)
- Olesen, O.V., Schou, M. & Thomsen, K.: Administration of lithium to rats by different routes. Neuropsychobiology 1976, 2: 134 - 138 (182)
- Pandey, G.N., Javaid, J.I., Davis, J.M. & Tosteson, D.C.: Mechanism of lithium transport in red blood cells. Physiologist 1976, 19: 321 (196)
- Pappius, H.M. & Elliott, K.A.C.: Water distribution in incubated slices of brain and other tissues. Can.J.Biochem. Physiol. 1956a, 34: 1007-1022 (27,28,41,76,78)
- Pappius, H.M. & Elliott, K.A.C.: Factors affecting the potassium content of incubated brain slices. Can.J.Biochem. Physiol. 1956b, 34: 1053-1067 (36,41)
- Pappius, H.M., Klatzo, I. & Elliott, K.A.C.: Further studies on swelling of brain slices. Can.J.Biochem.Physiol. 1962, 40: 885-898 (31,75,78,80)
- Pappius, H.M., Rosenfeld, M., Johnson, D.M. & Elliott, K. A.C.: Effects of sodium-free media upon the metabolism and the potassium and water contents of brain slices. Can.J. Biochem.Physiol. 1958, 36: 217-226 (19,157,200)
- Parker, R.E.: Introductory statistics for biology. The Institute of Biology's studies in biology no. 43. Edward Arnold, London 1973 (74,91)
- Partridge, L.D. & Thomas, R.C.: Effect of intracellular lithium on snail neurones. Nature (Lond.) 1974, 249: 578-580 (133)
- Paul, H.-A., Pilz, H. & Munz, E.: Der lithiumgehalt im liquor cerebrospinalis bei therapie mit lithiumsalsen. Nervenarzt 1973, 44: 210-211 (181,191,192)

- Phelps, C.F.: The physical properties of inulin solutions. Biochem.J. 1965, 95: 41 - 47 (75)
- Ploeger, E.J.: The effects of lithium on excitable cell membranes. The influence on the ATPase of homogenates of the non-myelinated nerve fibres of the rat. Arch.Int.Pharmacodyn.Ther. 1974a, 210: 374 - 382 (128)
- Ploeger, E.J.: The effects of lithium on excitable cell membranes. On the mechanism of inhibition of the sodium pump of non-myelinated nerve fibres of the rat. Eur.J.Pharmac. 1974b, 25: 316-321 (131)
- Ploeger, E.J. & Hertog, A. den: The effects of lithium on excitable cell membranes.II. The effect on the electrogenic sodium pump of non-myelinated nerve fibres of the rat. Eur.J.Pharmac. 1973, 21: 24 - 29 (128)
- Pradhan, N., Channabasavanna, S.M., Dwarkanath, B.S. & Talekar, S.V.: Fick's diffusion model for kinetics of lithium. IRCS Med.Sci. 1977, 5: 278 (109)
- Prockop, L.D. & Marcus, D.J.: Cerebrospinal fluid lithium: passive transfer kinetics. Life Sci. 1972, 11: 859-868 (191,202)
- Rall, D.P., Oppelt, W.W. & Patlak, C.S.: Extracellular space of brain as determined by diffusion of inulin from the ventricular system. Life Sci. 1962, 2: 43 - 48 (192)
- Richelson, E.: Lithium ion entry through the sodium channel of cultured mouse neuroblastoma cells: a biochemical study. Science 1977, 196: 1001-1002 (131,132,135,199)
- Ringer, S.: A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J.Physiol.(Lond.) 1883, 4: 29-42 (18)
- Robins, E., Smith, D.E., Eydt, K.M. & McCaman, R.E.: The quantitative histochemistry of the cerebral cortex - II. Architectonic distribution of nine enzymes in the motor and

- visual cortices. J. Neurochem. 1956, 1: 68 - 76 (31)
- Robinson, J.D.: Mechanisms by which Li^+ stimulates the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase. Biochim. Biophys. Acta 1975, 413: 459 - 471 (128,129)
- Scheffé, H.: A method for judging all contrasts in the analysis of variance. Biometrika 1953, 40: 87 - 104 (91)
- Schou, M.: Biology and pharmacology of the lithium ion. Pharmac. Rev. 1957, 9: 17 - 58 (24)
- Schou, M.: Lithium studies. 3. Distribution between serum and tissues. Acta Pharmac. Tox. 1958, 15: 115 - 124 (19)
- Schou, M.: The biology and pharmacology of lithium: a bibliography. Psychopharmac. Bull. 1969, 5(4): 33 - 62 (24)
- Schou, M.: A bibliography on the biology and pharmacology of lithium - appendix I. Psychopharmac. Bull. 1972, 8(4): 36 - 62 (24)
- Schou, M.: A bibliography on the biology and pharmacology of lithium - appendix II. Psychopharmac. Bull. 1976a, 12(1): 49 - 74, 12(2): 69 - 83 & 12(3): 86 - 99 (24)
- Schou, M.: Bibliography on the biology and pharmacology of lithium. 4. Neuropsychobiol. 1976b, 2: 161 - 191 (17,24)
- Schou, M.: Bibliography on the biology and pharmacology of lithium. 5. Neuropsychobiol. 1978a, 4: 40 - 64 (24)
- Schou, M.: The range of clinical uses of lithium. In Johnson, F.N. & Johnson, S. eds. Lithium in medical practice. M.T.P. Press, Lancaster, U.K. 1978b, p.p. 21 - 39 (17)
- Schou, M.: Bibliography on the biology and pharmacology of lithium. 6. Neuropsychobiol. 1979, 5: 241 - 265 (24)
- Schou, M., Juel-Nielsen, N., Strömberg, E. & Voldby, H.: The treatment of manic psychoses by the administration of lithium salts. J. Neurol. Neurosurg. Psychiat. 1954, 17: 250 - 260 (17,191)
- Schousboe, A. & Hertz, L.: Effects of potassium on indicator

- spaces and fluxes in slices of brain cortex from adult and new-born rats. J.Neurochem. 1971, 18: 67-77 (75)
- Schuberth, J., Sundwall, A., Sörbo, B. & Lindell, J.-O.: Uptake of choline by mouse brain slices. J.Neurochem. 1966, 13: 347 - 352 (111,200)
- Sershen, H. & Lajtha, A.: The distribution of amino acids, Na^+ and K^+ from surface to centre in incubated slices of mouse brain. J.Neurochem. 1974, 22: 977 - 985 (31,173,198)
- Skou, J.C.: Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol.Rev. 1965, 45: 596-617 (127,128,134)
- Smith, D.F. & Balagura, S.: The effect of lithium chloride on the electrolyte composition of cerebrospinal fluid of the rat. Physiol.Behav. 1972, 9: 261-262 (182,190)
- Smith, I.C.H.: Lithium, sodium and potassium fluxes in frog skeletal muscle. J.Physiol.(Lond.) 1974, 242: 99P - 101P (128,129)
- Solomon, A.K.: Compartmental methods of kinetic analysis. In Comar, C.L. & Bronner, F. eds. Mineral metabolism 1 A. Academic Press, New York 1960, p.p. 119-167 (73,167)
- Stahl, W.L. & Swanson, P.D.: Calcium movements in brain slices in low sodium or calcium media. J.Neurochem. 1972, 19: 2395-2407 (133)
- Steel, R.G.D.: Answer to query: Error rates in multiple comparisons. Biometrics 1961, 17: 326-328 (90)
- Stein, W.D.: The movement of molecules across cell membranes. Academic Press, New York & London 1967 (24)
- Sunderman, F.W.: Studies in serum electrolytes. X. The water of serum and factors for the calculation of the molality of a solute in serum from the measurement of the specific gravity. J.Biol.Chem. 1936, 113: 111-115 (72)
- Thellier, M., Stelz, T. & Wissocq, J.C.: Detection of stable

- isotopes of lithium or boron with the help of a (n, α) nuclear reaction. Application to the use of ^6Li as a tracer for unidirectional flux measurements and to the micro-localization of lithium in animal histologic preparations. Biochim. Biophys. Acta 1976, 437: 604 - 627 (21)
- Thomas, R.C.: Membrane current and intracellular sodium changes in a snail neurone during extrusion of injected sodium. J. Physiol. (Lond.) 1969, 201: 495 - 514 (131)
- Thomas, R.C., Simon, W. & Oehme, M.: Lithium accumulation by snail neurones measured by a new Li^+ -sensitive micro-electrode. Nature (Lond.) 1975, 258: 754 - 756 (133,175,178)
- Thomsen, K. & Olesen, O.V.: Long-term lithium administration to rats. Lithium and sodium dosage and administration, avoidance of intoxication, polyuric control rats. Int. Pharmacopsychiat. 1974, 9: 118 - 124 (182)
- Tobin, T., Akera, T., Han, C.S. & Brody, T.M.: Lithium and rubidium interactions with sodium- and potassium-dependent adenosine triphosphatase: a molecular basis for the pharmacological actions of these ions. Molec. Pharmac. 1974, 10: 501 - 508 (128,129)
- Tower, D.B.: Ouabain and the distribution of calcium and magnesium in cerebral tissues in vitro. Exp. Brain Res. 1968, 6: 273 - 283 (132)
- Umbreit, W.W., Burris, R.H. & Stauffer, J.F.: Manometric techniques, 3rd ed. Burgess, Minneapolis 1957 (37,38,48)
- Ussing, see: Koefoed-Johnson & Ussing
- Varon, S. & McIlwain, H.: Fluid content and compartments in isolated cerebral tissues. J. Neurochem. 1961, 8: 262 - 275 (27,29,30,31,35,41,75,77)
- Warburg, O.: Versuche an überlebendem carcinomgewebe (methoden). Biochem. Zeitschr. 1923, 142: 317 - 333 (18,30,34,39)
- Warburg, O. ed.: Über den stoffwechsel der tumoren. Springer,

Berlin 1926 (19)

- Watanabe, S., Taguchi, K., Ebara, T., Iguchi, K. Otsuki, S.: Lithium concentration in cerebrospinal fluid of affective psychotic patients treated with lithium carbonate and its clinical response. Folia Psychiatr. Neurol. Jap. 1973, 27: 299 - 303 (181,191,192)
- Wespi, H.H.: Active transport and passive fluxes of K, Na, and Li in mammalian non-myelinated nerve fibres. Pflügers Arch. 1969, 306: 262 - 280 (178)
- Widdicombe, J.H.: Ouabain- sensitive ion fluxes in the smooth muscle of the guinea-pig's taenia coli. J. Physiol. (Lond.) 1977, 266: 235 - 254 (131)
- Wilkinson, G.N.: Statistical estimations in enzyme kinetics. Biochem. J. 1961, 80: 324 - 332 (161)
- Winter, C.G. & Christensen, H.N.: Migration of amino acids across the membrane of the human erythrocyte. J. Biol. Chem. 1964, 239: 872 - 878 (164)
- Wraae, O., Hillman, H. & Round, E.: The uptake of low concentrations of lithium ions into rat cerebral cortex slices and its dependence on cations. J. Neurochem. 1976, 26: 835 - 843 (40,43,80,81,85,86,109,125)
- Zajčik, V.E., Stepanenko, V.F. & Rjabuchin, Ju.S.: Zur quantitativen neutronenaktivierungs-autoradiographie von Li^6 in biopräparaten mit hilfe von nitrozellulosefilm. Radiobiol. Radiother. (Berl.) 1972, 13: 691 - 703 (21)

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